SKELETAL ADAPTATION TO WHOLE BODY VIBRATION IN GROWING PIGS AND YEARLING HORSES

A Dissertation

by

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ABSTRACT

Two animal models, growing pigs (n = 26) and yearling horses (n = 20), were used to test the hypothesis that whole body vibration (WBV) would improve bone density and composition. Digital radiographs with an aluminum step wedge were used to determine bone density in terms of radiographic bone aluminum equivalency (RBAE). Serum biomarkers of bone formation (osteocalcin, OC) and bone resorption (carboxy-terminal collagen crosslinks, CTX-I) were determined as measures of bone cell actibvity. The effect of dietary calcium (Ca) and phosphorus (P) on bone was also tested in the pig study.

The maximum RBAE values for the medial or lateral cortices of the left third metacarpal bone were not affected by WBV in either the pig or horse models. Although there was not a statistically significant difference between vibrated pigs and horses and their respective controls, horses that were vibrated tended to have increased ($P = 0.062$) maximum RBAE values for the lateral cortices compared to controls. Pigs fed a diet with adequate concentrations of Ca and P tended to have increased RBAE max values for the medial and lateral cortices compared to those fed a diet with deficient concentrations of Ca and P. Mean RBAE max values for medial cortices increased (linear, $P = 0.028$) in pigs from d 0 to 60. Mean RBAE max values for the lateral cortices had a marginally significant increase (quadratic, $P = 0.084$). from d 0 to 60.

Horses and pigs receiving vibration treatment had decreased CTX-1 concentrations ($P = 0.003$ and $P = 0.044$, respectively) compared to the non-vibrated control group. The decreased CTX-I concentrations observed may be the result of an adaptive response of modeling bone to whole body vibration.

Mean serum concentrations for CTX-1 and OC increased (quadratic, $P = 0.0002$ and linear, $P = 0.001$, respectively) in horses from d 0 to 120 indicative of a measured bone turnover response. Increased CTX-I was likely the result of housing in individual stalls which could have contributed to increased bone resorption, as characterized by osteopenia during immobilization. Pigs fed a diet with adequate Ca and P had decreased concentration of OC from d 0 to 30, and then increased concentrations from d 30 to 60.

Whole body vibration treatment did elicit a response in the trabecular bone parameters trabecular number (TbN.) and trabecular separation (Tb.Sp) in pigs. Those that were vibrated had lower Tb.N values and higher Tb.Sp values, suggestive of bone resorption, and WBV did not significantly change any cortical bone parameters.

Normal physiological responses of bone to a low Ca, P diet were observed in this study. Although WBV did not elicit an osteogenic response, indications of an early local adaptive response were observed. The frequency and amplitude of WBV applied in this study was likely sufficient to elicit a bone remodeling response, but the duration of the study may not have captured the full cycle.

DEDICATION

This manuscript is dedicated to all the people that have inspired me to continually learn and grow in what you do not know.

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NOMENCLATURE

BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMU	Basic Multicellular Unit
BV/TV	Bone Volume / Total Volume
Cort. CSA	Cortical Cross-Sectional Area
Cort. Th	Cortical Thickness
CT	Computed Tomography
CTX-I	Carboxy-terminal Collagen Crosslinks
DXA	Dual Energy X-ray Absorptiometry
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
OC	Osteocalcin
PTH	Parathyroid Hormone
QCT	Quantitative Computed Tomography
RANK	Receptor Activator of Nuclear Factor kappa B
RANKL	Receptor Activator of Nuclear Factor kappa B Ligand
RBAE	Radiographic bone aluminum equivalency
TbTh	Trabecular Thickness
TbN	Trabecular Number
TbSp	Trabecular Spacing
WBV	Whole Body Vibration

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CHAPTER I

INTRODUCTION

Healthy bones, as assessed by adequate bone mass and strength. Underpin the health of an individual (animal or human). The mass and strength of bone can be positively (or negatively) affected by mechanical loading of the skeleton. Whole body vibration (WBV) is a relatively easy therapy that has the potential to load parts of the skeleton to achieve these adaptive responses. The body performs many activities that increase load and strains experienced by the skeleton. The transmission of load to bone is sensed by bone embedded cells (osteocytes) that respond accordingly. Load is transmitted to the skeleton by all manners of activity, walking, running and for this study vibration. Exogenous methods, such as WBV, that are practical and easily implemented, can potentially increase bone mass and strength, being beneficial for animals and humans alike.

In the equine athlete, WBV could potentially be utilized to stimulate bone adaptation to ensure the skeletal system can withstand intense training, potentially resulting in decreased incidence of lameness, break down, and/or skeletal failure. In humans, WBV has been of great interest in the medical community as a means to counteract the bone loss from post-menopausal osteoporosis. The National Osteoporosis Foundation reports that approximately 54 million Americans have osteoporosis and low bone mass. This has attributed to 2 million broken bones and $19 billion in related costs every year (National Osteoporosis Foundation, 2015). In addition, numerous studies have reported the positive effects of WBV in patients with cerebral palsy, Duchenne's muscular dystrophy, and pediatric leukemia, where osteopenia from disuse (unloading) is a major

debilitating complaint (Ward et al., 2004; Rauch, 2009). Thus, WBV is considered a promising therapy to counteract bone loss in humans.

Bone research in humans and horses is largely limited to in vivo studies and can be complicated by mammal size and expense related to testing, therefore other animal models are utilized to provide supporting evidence of mammalian responses. The pig is a good model for both human and horse bone research due to its more manageable size, similar bone qualities, and relative acceptance of harvest for ex vivo testing. Therefore, a preliminary study was conducted with pigs to utilize techniques that yield more detailed insights on bone metabolism and microarchitecture.

Additionally, skeletal integrity is vital for economic sustainability of swine operations. Pigs in the production cycle must remain sound or be culled to minimize economic loss. Many factors contribute to bone quality and strength for soundness, with adequate mineral intake of high priority. Calcium and phosphorus are two important dietary mineral considerations for adequate skeletal growth and maintenance. A balance must be achieved to avoid excess or deficiency when feeding minerals such as Ca and P for skeletal development, therefore this study also tested the effects of decreased dietary Ca and P levels.

Despite the prevalence of anecdotal claims that WBV therapy improves physiological functions or increases bone density, conclusive evidence does not exist to substantiate these claims especially in the young, growing animal. The research presented in this □issertation was designed to assess the purportedly positive effects of WBV on bone metabolism, bone microarchitecture, and bone mass in growing pigs and yearling horses.

CHAPTER II

LITERATURE REVIEW AND SUMMARY

LITERATURE REVIEW

Bone Biology

The skeleton is complex organ that consists of the cartilaginous joints, the calcified cartilage of growth plate, marrow cavity, cortical bone, and cancellous bone. The mammalian skeleton is composed of a large number of bones that are categorized into four groups: long bones, short bones, flat bones, and irregular bones (Bilezikian et al., 2008). Bone is a vital living tissue of the body and is constantly undergoing remodeling to maintain bone health and strength. During growth, bone undergoes a substantial amount of modeling to ultimately reach genetic potential of size and mass (Bilezikian et al., 2008). The skeleton functions to provide structural support for the body, is a mineral depot to maintain mineral homeostasis, and support hematopoiesis within the marrow spaces (Taichman et al., 2005).

Long bones provide structure, strength, and mobility to the body. They are composed of a diaphysis, a tubular shaft, that spreads into a metaphysis on each end that is wider than the shaft. The metaphysis and epiphyses are separated by the growth plate. The diaphysis is composed of dense compact bone with a ratio of cortical bone to trabecular bone of 95:5. The metaphysis and epiphysis are primarily composed of trabecular bone surrounded by a thin shell of cortical bone. surrounding a central medullary cavity. As a whole, the skeleton is composed of 80% cortical bone and 20% trabecular bone (Hadjidakis and Androulakis, 2006). Cortical bone is dense and attributes to much of the strength of the skeleton. Trabecular bone takes more the form of a sponge with open spaces

and a network of trabecular plates and rods. Although very different in overall structure, both the cortical and trabecular bone of larger animals (dogs, pigs, horses, humans) are composed of osteons (Clarke, 2008).

Cortical bone is maintained and remodeled by the Haversian system. Cortical bone is typically less metabolically active than trabecular bone (Clarke, 2008; Kim and Park, 2013). Cortical bone porosity is typically very low, however porosity does tend to increase with aging. Remodeling activity takes place on both the outer periosteal surface and the inner endosteal surface. Appositional growth largely takes place at the periosteum. Trabecular osteons are called packets. Cortical and trabecular bone typically form in a lamellar pattern (Bilezikian et al., 2008), which gives bone its strength and rigidity.

Bone undergoes substantial longitudinal and radial growth during growth and development. Longitudinal growth begins at the growth plate as cartilage that eventually proliferates into the epiphysis and diaphysis and becomes mineralized to form new bone. Bone modeling changes the shape of bones to better withstand mechanical forces placed on the skeleton (Seeman, 2009). Bone resorption and formation are not closely coupled in modeling of bone as formation predominates during growth (Hillam and Skerry, 1995). In healthy individuals, modeling predominately takes place during growth and development and becomes minimal in adulthood (Seeman, 2009).

The skeleton is continually renewed by a process of bone remodeling, which is resorption and formation carried out by osteoclasts and osteoblasts, respectively. Remodeling is continuous throughout life, serving to replace old bone or specific areas of micro damaged bone with new bone to sustain a bone balance (Seeman, 2009). Bone remodeling begins from a state of quiescence and is carried out by the coupled activity of

osteoclasts and osteoblasts in the basic multicellular unity (BMU) (Fig 1, step 1) (Hadjidakis and Androulakis, 2006). Bone resorption is mediated by osteoclasts and takes approximately 2 to 4 wk during each cycle (Fig 1, step 2). Osteoclast formation, activation, and resorption activity is regulated by numerous factors including the ratio of receptor activator of NF-κB ligand (RANKL) to osteoprotegerin (OPG), IL-1 and IL-6, colony-stimulating factor (CSF), parathyroid hormone, 1,25-dihydroxyvitamin D, and calcitonin (Boyle et al., 2003; Blair and Athanasou, 2004). Osteoclasts secrete hydrogen ions into a resorption compartment created by integrin receptors linking the osteoclast membrane to the bone lining. As the mineral component of the bone matrix is dissolved by H^+ ions, cathepsin K digests the matrix, which is mostly composed of type I collagen (Boyle et al., 2003). Resorption pits made by osteoclasts on the surface of trabecular bone are called Howship's lacunae and in cortical bone the BMU forms a cylindrical canal creating the Haversian canals. (Eriksen, 1986; Reddy, 2004). Bone resorption then transitions to formation through a reversal phase that can last up to 4 or 5 weeks and involves monocytes, osteocytes, and pro-osteoblasts (Fig 1, step 3) (Hadjidakis and Androulakis, 2006). Bone formation takes approximately 4 to 6 mo to complete. Osteoblasts secrete bone matrix in the void created by osteoclasts (Fig 1, step 4). Ultimately, some osteoblasts become trapped in their own bone matrix giving rise to osteocytes. Osteocytes are the most abundant cells in bone and they communicate through an extensive canalicular network that connects them to bone surface lining cells, osteoblasts, and each other (Turner et al., 2002; Buenzli, 2015). Therefore, osteocytes are thought to act as mechanosensors, instructing osteoclasts and osteoblasts in the remodeling process (Burger et al., 2003). Newly formed osteoid (unmineralized bone matrix) is mineralized to form bone resulting

in the completion of the remodeling cycle (Hadjidakis and Androulakis, 2006). Bone remodeling is essentially the same for cortical and trabecular bone (Clarke, 2008).

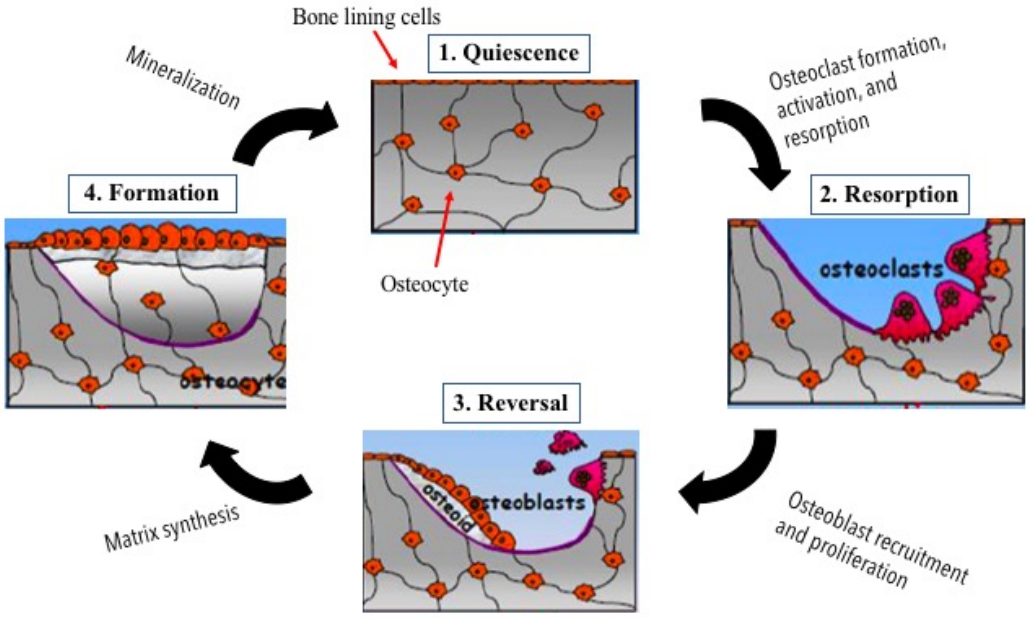

Figure 1. Schematic of bone remodeling cycle.

Horse Skeleton

Survival of the horse is dependent upon offspring having the ability to stand on a sound skeletal structure and develop rapidly. Prior to birth, the third metacarpal bone of the equine fetus changes from circular to conical to allow for immediate weight bearing after birth (Oikawa et al., 1991). Due to the proximal placement of bulky limb musculature, the equine appendicular skeleton is designed for minimizing energy expenditure during locomotion, even at a very young age. This design, however, can be to the detriment of the performance horse whom is plagued with musculoskeletal injuries (Hodgson et al., 2014).

The highest readings were obtained in the proximal shaft where the cortex was thickest. The velocity values gradually decreased towards the distal end where the cortex was thinner, particularly in animals less than 12 mo old.

Many studies of weanling and yearling horses have shown an increase in bone mineral content up to the first year and a half of life, with increasing mineralization of the skeleton occurring with maturation (Buckingham and Jeffcott, 1987; Raub et al., 1989; McCarthy and Jeffcott, 1992; Nielsen et al., 1997; Reichmann et al., 2004). Cross-sectional area of the third metacarpal bone increases with age (Jeffcott and McCartney 1985; Nunamaker et al. 1989; Buckingham et al. 1992), with a sharp increase between 1d and 1yr of age (El Shorafa et al., 1979).

Studies by Lawrence et al. (1994) and El Shorafa et al. (1979) have provided insight to properties pertaining to quality and quantity of the third metacarpal bone over time. Lawrence et al. (1994) used 25 horses ranging from 1 d to 27 yr old and El Shorafa et al. (1979) used 41 horses ranging from 1 d to 33 yr old. Lawrence et al. (1994) found maximum BMC was reached at 6.0 ± 1.8 yr of age and 76% of maximum BMC was achieved by 1 yr of age. Maximum breaking load was reached at 4.6 ± 1.8 yr of age and was highly correlated to BMC ($r^2 = 0.92$). Breaking strength peaked at 6.3 ± 1.2 yr of age and it was well correlated with BMC ($r^2 = 0.84$). By 1 yr old, 85% of maximum breaking strength was achieved. El Shorafa et al. (1979) reported that the metacarpal bone reaches maximum ash content, cortex area and failure stress resistance at age 4 to 7 years. Ash content reaches a maximum at 4 yr of age, which is maintained through age 7 yr, then begins to decline (El Shorafa et al., 1979). As detected by ultrasound, horses have thicker

cortex at the proximal shaft of the metacarpal bone and thickness decreases towards the distal end, especially in horses under the age of 1 yr (Jeffcott and McCartney, 1985).

The third metacarpal bone and the twelfth rib have similar calcium levels, but differ significantly in phosphorus, dry fat-free ash percentage, and ash as a percentage of wet weight (Cooper et al., 2001). Calcium, phosphorus and magnesium in bone ash of the third metacarpal ranges from 35 to 39, 14 to 17 and .32 to .85%, respectively. The range of calcium to phosphorus ratio is 2.1 to 2.6 (El Shorafa et al., 1979).

Pig Skeleton

Pigs have been extensively used in biological models for humans biomedical research due to their distinct similarities in anatomy, physiology, metabolism and nutrition (Bustad, 1966; Douglas, 1972; Pong, 1978; Miller and Ullrey, 1987; Swindle et al., 2012). There are also some important similarities in bone composition between humans and the pig (Dickerson, 1962).

Growth in the pig requires a skeletal structure that adapts to growth in muscle and fat very quickly to maintain structurally integrity (Tanck et al., 2001). Trabecular bone adapts to loading by altering both density and architecture (Bilezikian et al., 2008). Load placed upon the pig skeleton increases gradually as size and weight increases, therefore eliciting trabecular bone adaptation. Growth plates of the proximal tibia were nearly closed at 104 wk old pigs in a study conducted by Tanck et al. (2001) to quantify trabecular bone adaptation to growth. The author equated 104 wk old pigs to be similar to about 15 yr old humans and 230 wk old pigs to about 30 yr old humans (Tanck et al., 2001).

From birth to 84 d of age (up to 31 kg live weight), growth of the long bones in the limb increase in shaft diameter more than length (Liu et al.). However, growth in diaphysis

length of the metacarpal bones is substantial during the growth period from 50 to 68 kg carcass weight whereas growth in thickness and density predominates from 68-92 kg (Cuthbertson and Pomeroy, 1962).

Bone ash is approximately 37.22% Ca and 18.88% inorganic P with an estimated C to P ratio of 1.97:1 (Eveleth, 1938; Ryan et al., 2011). Long bones such as the femur and rib contain 22.3% and 20.4% Ca respectively, in pigs (Field et al., 1974).

In the context of bone studies, pigs have some attributes that make them a good model for human research (Douglas, 1972; Miller and Ullrey, 1987; Swindle et al., 2012). In a comparative study by Aerssens et al. (1998), the dog and pig most closely aligned with human bone mineral content (BMC), volumetric bone mineral density (vBMD), and fracture stress values compared to the cow and sheep. Ash content of the cortical bone was similar in human, dog, and pig and of trabecular bone was similar in human, dog, pig, and sheep (Aerssens et al., 1998).

Bone Remodeling Biomarkers

The skeleton is continually renewed by a process of bone turnover, which is resorption and formation carried out sequentially by macrophage-derived osteoclasts and mesenchymal-derived osteoblasts, respectively (Bilezikian et al., 2008). Bone turnover markers give insight to these processes, risk of fracture, and response to treatments and can be complementary to other bone testing measures (Hlaing and Compston, 2014).

Bone turnover is initiated by the resorption of bone by osteoclasts eroding bone mineral surfaces (Bilezikian et al., 2008). Osteoclasts attach to the bone surface, form a detailed and elaborate ruffled border membrane and then secrete a mixture of acid and proteases onto the bone surface to degrade the collagen into fragments and liberate mineral

and embedded growth factors (Blair and Athanasou, 2004). The measurement of collagen breakdown products, including hydroxyproline (OHP), hydroxylysine glycosides, and the collagen cross-links, provides a measure of the extent of bone resorption and insight to the bone turnover process (Calvo et al., 1996; Allen, 2003). Other useful biomarkers, but not as widely used, include enzymes, such as tartrate-resistant acid phosphatase (TRAcP) and cathepsin K, secreted by osteoclasts and required for the degradation of collagen type I (Cremers et al., 2008; Vassalle and Pagani, 2016).

Most bone resorption markers are indicators of collagen breakdown during osteoclast activity. Hydroxyproline (OHP) has historically been an important marker for resorption and measures levels in the urine. However, OHP is present in nearly all tissues and lacks specificity for bone, rendering it less widely used in current research (Cremers et al., 2008). Collagen cross-links, pyridinoline (PYD) and deoxypyridinoline (DPD), are predominately in skeletal tissue and measurement in urine as a sensitive index of the extent of bone resorption (Seibel et al., 1992). However, DPD is more prevalent in bone and dentin (Eyre et al., 1984). The amino- and carboxy-terminal cross-linked telopeptides of type I collagen (NTX-I and CTX-I, respectively) are two widely used bone resorption markers that have relatively high sensitivity and specificity for the degradation of type I collagen. However, choosing to measure CTX-I or NTX-I must be determined with great care to deduce the most relevant information, as each marker varies with specific bone metabolism, diseases and assays (Herrmann and Seibel, 2008). Galactosyl hydroxylysine is a modified amino acid of collagen and is relatively specific to bone collagen degradation (Krane et al., 1977) however, the lack of commercial immunoassay has resulted in a lack of incorporation of this marker into many bone studies (Cremers et al., 2008).

Other useful biomarkers as indicators of bone resorption involve the enzymatic degradation of bone by the osteoclasts. Enzymes such as TRAcP are located in the ruffled border of the osteoclast membrane and in the bone resorption lacunae. Tartrate-resistant acid phosphatase activity has been reported to be indication of bone resorption rates (Yam, 1974). However, its distinction from other tissue acid phosphatases in serum has yet to be elucidated and its stability in serum is poor, therefore creating challenges with its use (Cremers et al., 2008). Cathepsin K is an enzyme located in the cytoplasm of osteoclasts and is secreted in the resorption lacunae to induce bone collagen degradation. It has appealing potential to measure bone degradation but is largely understudied for clinical use and assays available lack sensitivity (Cremers et al., 2008).

Bone resorption activity is followed by bone formation. Osteoblasts secrete new bone matrix (osteoid) that gradually fills in the resorptive cavity made by osteoclasts. Biomarkers involved in osteoblastic activity can be measured to determine level of bone formation. They include the propeptides of type I collagen, osteocalcin (OC), and bone-specific alkaline phosphatase (BALP) (Weaver et al., 1997).

Propeptides of type I collagen with extensions amino (N)- and carboxyl (C)-terminal are cleaved during collagen biosynthesis resulting in the C propeptide (PICP) and amino-terminal propeptide (PINP). As with any collagen type I related markers, other contributions from soft tissue synthesis of type I collagen could potentiate differences in actual PICP and PINP levels. However, the rate of collagen turnover in bone is faster than in other tissues and therefore any changes in concentration could be assumed to reflect bone collagen synthesis (Cremers et al., 2008). Good correlations between serum PICP and

bone formation have been demonstrated though (Hassager et al., 1991; Eriksen et al., 1993).

Osteocalcin is a small protein synthesized by osteoblasts. It is a sensitive and specific marker for measuring bone formation from osteoblastic activity (Brown et al., 1984; Charles et al., 1992; Weaver et al., 1997). In humans, a circadian rhythm in OC levels has been characterized by a decline during the morning to a low around noon, followed by a rise in the afternoon and early evening, and reached a peak nocturnally (Gundberg et al., 1985). Seasonal changes have also been defined with higher levels of OC in the winter and spring compared to summer and fall. This effect could be attributed to subclinical vitamin D deficiency during the winter period (Thomsen et al., 1989; Douglas et al., 1996; Woitge et al., 1998). Therefore, consideration must be given for timing of sample collections. Osteocalcin is not released during bone resorption and therefore measured levels can confidently be interpreted as osteoblastic activity in bone formation (Price et al., 1981).

Alkaline phosphatase is an enzyme and its two most common organ sources are liver and bone. Techniques specific to each isoform have been developed to distinguish from which source the ALP is derived. There is great variation from individual to individual in ALP levels (Crofton, 1982) and BALP levels are affected by age, gender, and hormonal status (Calvo et al., 1996).

Bone turnover biomarkers can be very helpful to substantiate other bone analyses. The markers most used in the human clinical settings include OC, BALP, PINP, and CTX-I (Bergmann et al., 2009; Brown et al., 2009). International Scientific Societies (IOF, IFCC, and NBHA) recommend the use of serum PINP and serum CTX-I in laboratory and

clinical applications for determining rate of bone formation and resorption, respectively, in osteoporosis. (Bergmann et al., 2009; Brown et al., 2009; Vasikaran et al., 2011; Bauer et al., 2012).

All the biomarkers in the aforementioned section have been validated and successfully used in human clinical settings. However, additional information is still needed to legitimize the use all these biomarkers in animals to quantity the relationship between serum and urine levels and bone turnover. With animals, information on biomarker synthesis and secretion response to criteria such as aging, exercise, disease, surgery, or medical treatment is lacking (Allen, 2003). Diurnal rhythms exist for serum OC and urinary PYD and DPD in adult horses, confirming that time of collection should be considered when using bone biomarkers (Lepage et al., 1991; Black et al., 1999).

When assessing bone turnover in the horse, serum and urine testing can be more sensitive, economical, and less invasive in comparison to more traditional techniques employed, such as radiography and bone biopsy. Over the last three decades, a minimal number of studies have been published on biomarker validation to quantify bone turnover in the horse. Biomarkers most widely accepted for use in the horse include OC, PICP, BALP, DPD, ICTP, and CTX (Lepage et al., 2001).

Post hoc analysis compiled by Nielsen et al. (2008) showed that osteocalcin corresponds strongly with estimates of bone quality in horses. Serum BALP and osteocalcin concentrations were measured in foals from birth to 112 d of age by Reller et al. (2003). High biological variability was observed between foals, but there tended to be no differences in bone formation markers between sex. Thoroughbred foals had higher osteocalcin and BALP concentrations than Quarter Horse foals. Differences in osteocalcin

concentrations have also been reported between draft and warmblood horses (Lepage et al., 1997). An inverse correlation between age and bone biomarkers PICP, ICTP, and BALP was observed by Price et al. (1995). Lepage et al. (1990) observed this same correlation between ALP, OC, and age up to 48 mo. The most significant changes in bone biomarkers happened over the first two years. In newborn foals, 92% of total ALP is attributed to BALP compared to approximately 20% in horses over 5 yr of age. A marked decrease in serum osteocalcin has been observed over the first 30 months of life (Lepage et al., 1990) and 18 months of life (Price et al., 2001) in the horse, indication that there is a significant slowdown in bone formation from birth to what is considered to be a mature adult horse. A positive correlation, as well, has been observed in BMC and OC and CTX-1 levels with age (Lepage et al., 1990; Fletcher et al., 2000; Reichmann et al., 2004; Donabédian et al., 2008). Osteocalcin has been shown to decrease with onset of industry race training (0-42 d) and then increase in the serum with continued training (42-112 d), which also followed RBAE values depicting BMC (Nielsen et al., 1998). Billinghurst et al. (2003) reported significantly higher percent changes from baseline in CTX-I serum levels for foals receiving forced exercise in comparison to those stalled or pastured, which would be suggestive of bone resorption. And finally, for the first 28 d of stalling, horses not exercised had decreased osteocalcin concentrations and increased urinary bone resorption marker DPD (Hoekstra et al., 1999), perhaps suggestive of a disuse response.

Over a 140-d period, Hoekstra et al. (2010) compared BMC and biochemical markers, OC and DPD, in stalled versus pastured yearling horses, and detected an increase in DPD in stalled horses. Bone mineral content of the third metacarpal also decreased in stalled horses. Both outcomes together would be indicative of decreased loading in horses

that are stalled and therefore expected increase in bone resorption (Hoekstra et al., 2010). Other bone resorption biomarkers have been studied in the horse with the most useful being type I collagen degradation molecules. Draft horses have higher serum ICTP levels than Warmblood horses whereas, serum OC levels are lower in Draft horses than in Warmblood horses (Lepagea et al., 1998). Levels of serum ICTP decrease with age in horses and the most significant changes occur during the first year of life (Price et al., 1995). Carboxy-terminal cross-linking telopeptide of type I collagen concentration was also inversely correlated with age (Carstanjen et al., 2004). As horses aged, serum levels of CTX-I decreased. However, in horses under a year old, CTX-I concentrations have been shown to significantly increase up to 11 mo old (Billinghurst et al., 2003). Horses from this study that had free exercise at pasture had less type I collagen degradation than those that received forced exercised or were box stalled with no exercise. No significant circadian variations in plasma CTX-I and serum osteocalcin concentrations in horses were detected by Carstanjen et al. (2004).

Nutritional Influence of Macro Minerals Ca and P on Bone

Calcium and phosphorus are important components of bone and vital for bone modeling and remodeling. They also play a vital role in intracellular and extracellular activities of the body. Calcium is essential for maintenance of nerve tissue, resting membrane potential, blood clotting mechanisms, and myocardial contraction. Calcium also regulates activity of many enzymes, microtubule assembly, generation of ATP, release of hormones and neurotransmitters and muscle cell contraction (Epstein et al., 1986; Littledike and Goff, 1987). Phosphorus is the second most abundant essential mineral in the body, next to Ca. It plays a major role in bone mineralization as most P is skeletal as

hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Approximately 10-20% of P is not in skeletal tissue and provides the structural framework for nucleic acids and is used in energy metabolism (Raina et al., 2012).

Blood serum Ca in the pig is on average 11.93 mg/dl. Bone ash is approximately 37.22% Ca and 18.88% inorganic P with an estimated C to P ratio of 1.97:1 (Eveleth, 1938; Ryan et al., 2011). Complex homeostatic mechanisms maintain blood Ca to mitigate hypocalcemia and hypercalcemia (Fig. 2). Some Ca leaves the body permanently, such as through urine and feces excretion, milk expulsion, and birth of the fetus. Calcium from these losses must be replenished by the diet or drawn from reserves in the body. Bone serves a mineral depot and is used to restore Ca levels in the blood and in most cases can restore homeostatic conditions. Calcium concentrations in the plasma are regulated by primarily three hormones: parathyroid hormone (PTH), 1, 25-dihydroxyvitamin D_3 (calcitriol), and calcitonin (CT). Parathyroid hormone is secreted by the parathyroid glands and normally in response to hypocalcemia. It signals the renal tubules to reabsorb Ca that would otherwise be excreted in the urine. Parathyroid hormone also stimulates the conversion of 25-hydroxy vitamin D into calcitriol in the kidney. Calcitriol also works to increase Ca plasma levels by stimulating dietary Ca uptake from the gastrointestinal tract, renal tubular reabsorption of Ca, and osteoblast release of RANKL to activate osteoclasts. Parathyroid hormone also works on bone to indirectly stimulate osteoclast activity and increase Ca release into the blood. Calcitonin is therefore a counter balance to PTH, regulating increases in Ca plasma levels. Calcitonin is secreted from the thyroid gland and acts on the kidney to increase urine excretion of Ca. Calcitonin also inhibits osteoclast resorption of bone (Littledike and Goff, 1987).

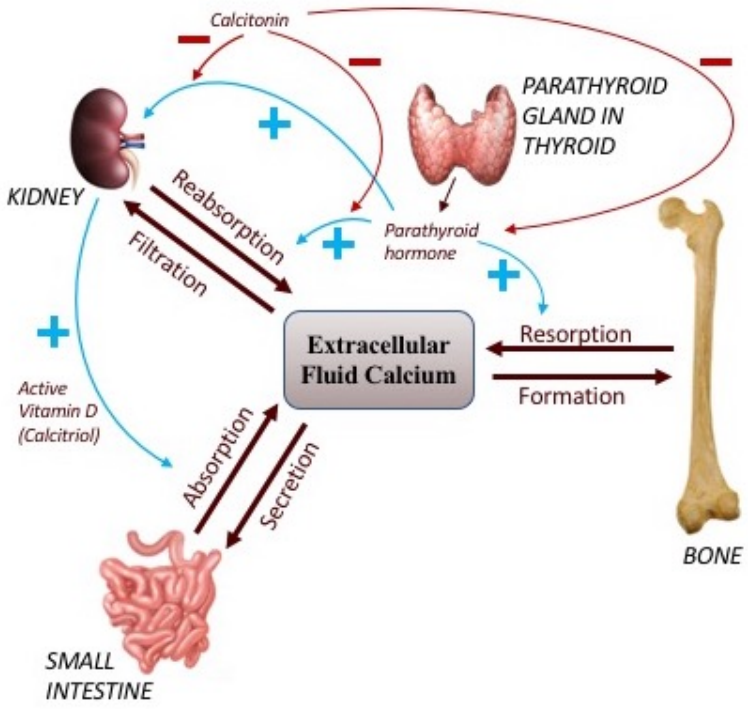

Figure 2. Schematic representation of Ca regulation by parathyroid hormone (PTH), calcitonin, and calcitriol (active Vitamin D).

Phosphorus is not as tightly regulated as Ca, but still can cause consequential disturbances if left unchecked. It is largely regulated by actions of the kidney. By PTH stimulation, excretion of P is increased through the renal tubules. Calcitriol's stimulation to increase bone resorption to release Ca from the bone also results in a release of P into the plasma (Schröder et al., 1996).

Calcium and P are two important diet considerations for adequate skeletal growth and maintenance. Dietary Ca and P are bound together in the bone at a constant ratio of 2.2:1 and the skeleton is a reserve for both Ca and P (Crenshaw et al., 2001). A balance must be achieved to avoid excess or deficiency when feeding minerals such as Ca and P for skeletal development (Doige et al., 1975). Excess mineral intake of P has resulted in decreased bone growth, skeletal material, and structural properties and decreased bone

strength in growing rats despite adequate Ca intake (Huttunen et al., 2006). High levels of Ca and P intake in growing pigs did not increase mineral accretion in bone and decreased bone resorption. Therefore Fernández (1995) concluded optimal mineral supply for normal bone development and turnover is more complicated than simply increasing mineral intake. Additionally, Wu et al. (2018) demonstrated that excess dietary Ca negatively affected growth performance and percentage bone ash of nursery pigs when diets were deficient in P. Deficient bone mineralization combined with an increased bone resorption was observed in growing pigs fed for 32 d on diets low and very low in Ca, at 0.4% and 0.1%, respectively (Eklou-Kalonji et al., 1999). Reducing digestible P in the diet from 3.7 to 3.0 g/kg for 28 d did not affect aBMD in metacarpal bones of pigs fed to an average end weight of 26.25 kg, but feeding the deficient diet for 56 d did result in a decreased aBMD. Reducing digestible P in the diet from 2.8 to 1.6 g/kg for 35 d decreased skeletal aBMD in the whole pig and individual limbs when fed to an average end weight of 102.43 kg (Ryan et al., 2011). Finally, increasing attention has been placed on the environmental impact of feeding excess P and economic loss, therefore identifying adequate feeding rates is important (Poulsen, 2000). Dietary P is the third most expensive ingredient after protein and energy. It typically is supplied by grain-based diets which provide P bound by phytates. This necessitates supplementing with non-renewable sources, such as inorganic P, which greatly increases feed costs (Cordell et al., 2009).

Diets deficient in Ca and P can predispose pigs to decreased bone quality and quantity, increasing the risk of bone fractures and resulting in economic loss. Bone mineral density and content was decreased in growing pigs fed diets deficient in P (Liesegang et al., 2002). Gonzalo et al. (2018) demonstrated that diets low in Ca and P decreased gain of

whole body BMC in the growing phase of pigs from 15 to 35 kg BW. Pigs on the control diet had a bone mineralization gain of 10.4 g/day in comparison to pigs fed a low Ca, P diet at 3.99 g/day. Interestingly, the same study reported a -0.014 g/day bone mineralization gain for vertebrae L2-L4 in pigs fed a low Ca, P diet in comparison to controls at 0.136 g/day for that same growing period. Overall, through four phases of growth (15-130 kg BW), on average, whole body bone demineralization was 23% and lumbar vertebrae was 30% in pigs at the conclusion of the depletion phase with low Ca, P diets. Vertebrae are composed of cortical and trabecular bone at a ratio of approximately 25:75 in comparison to long bones, such as the femoral head at 50:50 and the radial diaphysis at 95:5. Trabecular bone is typically more metabolically active than cortical bone in maintaining mineral homeostasis (Clarke, 2008). Trabecular bone also has a greater surface-to-volume ratio that is 10 times higher than cortical bone making it more sensitive to early biochemical changes in bone metabolism (Kim and Park, 2013). Therefore, a more pronounced effect of demineralization would be expected for trabecular bone, which was demonstrated by Gonzalo et. al. (2018). Additionally, greater chemical composition changes were observed in the vertebrae of miniature piglets fed diets low in Ca and P in comparison to their tibia and cranium (Schanler et al., 1991).

Several studies have characterized skeletal changes due to calcium and phosphorus deficiencies in growing swine. Calcium deficiency has shown to induce osteoporosis, hyperparathyroidism, osteodystrophy, and decreased BMC in growing pigs (Storts and Koestner, 1965; Shaw et al., 2006; Gonzalo et al., 2018) and rickets in piglets (Miller et al., 1962). It has also been demonstrated that P deficiency can lead to osteoporosis in growing pigs (Bayley and Thomson, 1969) and rickets in piglets (Miller et al., 1962).

Bone Response to Mechanical Loading

The skeleton is highly sensitive to mechanical loading and by remodeling, it structurally adapts to increase bone mass and strength (Nilsson and Westlin, 1971; Williams et al., 1984). Wolff (1892) first proposed the alterations of the internal architecture from "stressing of the bones." The mechanisms of the bone remodeling cycle are tightly coupled between bone resorption and formation, and defects such as microfractures are repaired by their coupling (Hadjidakis and Androulakis, 2006). Functional adaptation to mechanical loading or reduced loading, as with disuse (Zerwekh et al., 2009), requires local regulation or "mechanostat" to meet loading demands on the bone (Frost, 1987; Sugiyama et al., 2010). Deformation of the bone from loading can be measured through strain. Osteocytes are the most abundant bone cells in the skeleton and have been identified as sensors and transductors of strain (Huiskes et al., 2000; Han et al., 2004; Buenzli, 2015). Osteocyte signaling induces bone formation through a complex syncytium formed with osteoblasts and lining cells (Huiskes et al., 2000; Han et al., 2004; Suva et al., 2005).

The appendicular skeleton of the horse undergoes tremendous strain especially during extreme locomotion (Biewener et al., 1983; Rubin et al., 2013). Functionally induced strain provides a means for translating demands from activity into a site-specific signal relevant to bone morphology (Rubin et al., 2013). Horses typically have four gaits that they use for locomotion including the walk, trot, canter, and gallop. Depending on the gait, strain is applied to the bones of the limb at different levels during movement. The third metacarpal bone (cannon bone) is often used to study strain in the horse due to its lack of muscle attachment and minimal soft tissue interference at the midshaft (Rubin et

al., 2013). The distribution of normal strain indicates that the third metacarpal is loaded in a combination of axial compression (Biewener et al., 1983) and bending (Gross et al., 1992). Rubin et al. (2013) found that cantering resulted in the greatest maximal compression strain on the cannon bone, which was a 2-fold increase from the walk. The posterior/lateral cortex was consistently exposed to the greatest magnitude normal and shear strain, while the anterior/medial cortex was consistently exposed to the lowest strain (Rubin et al., 2013). Studies have shown an increase in bone mineral content of the medial and lateral cortices compared to the dorsal and palmar, indicating a greater strain perceived in those locations (Hiney et al., 2004). Maximum compressive strains were located within the posterior/medial quadrant, while tensile strains were found in the anterior/lateral aspect of the bone of one 5 yr old Thoroughbred (Gross et al., 1992). Bigot et al. (1996) reported that specimens taken from birth to 4 yr of age, from the lateral and medial cortices at mid-diaphysis of the metacarpal bone, had greater average bending strength, average Young's modulus, and average yield stress than the cranial cortices. The caudal cortices gave the lowest values in that study (Bigot et al., 1996). Weanlings exercised at a medium trot for up to 20 min, 5 d/wk tended to have an increased radiographic bone density of the medial cortices of the third metacarpal bone (Raub et al., 1989). At mid-diaphysis, the third metacarpal bone appears to be designed to resist axial compression and mediolateral bending, as it has a greater stiffness than the anterior/posterior plane, and exhibits uniform resistance to torsion along its length (Piotrowski et al., 1983). Increasing bone strength and its resistance to strain has the possibility to reduce skeletal failure and therefore decrease wastage in the equine industry (Verheyen and Wood, 2010).

The response of bone to exercise has been documented in foals (Raub et al., 1989; Cornelissen et al., 1999; Firth et al., 1999) yearlings (Schryver, 1978; McCarthy and Jeffcott, 1992), and horses undergoing training (Firth et al., 2005; Murray et al., 2007; Firth et al., 2011) The impact of confinement on bone in young horses has been studied to some extent. Young horses entering training are typically stalled and therefore the repercussion of disuse on the skeleton is of great interest to the equine industry. Many have demonstrated that confining a horse under the age of 2 yr to a stall without exercise results in increased bone resorption and therefore decreased bone mineral content, decreased bone formation, and delayed musculoskeletal development (Mäenpää et al., 1988; Hoekstra et al., 1999; Bell et al., 2001; Barneveld and Weeren, 2010).

In humans it has been demonstrated that loading of the skeleton, such as with exercise, can increase bone mass and strength (Nilsson and Westlin, 1971; Williams et al., 1984). The same can be concluded for horses (Jeffcott et al., 1987; Warden et al., 2004; Nielsen et al., 2008). The intensity of exercise influences the level of bone response. Long, slow exercise, such as with endurance training, has not shown to increase bone mineral content as determined by radiographic bone aluminum equivalency (RBAE) in 2 yr olds (Spooner et al., 2008). This study, however, did not balance for gender, having all fillies in the control group and all geldings in the treated group. Even so, numerous publications examining the impact of exercise on bone mineral content have validated no effect of sex on results (Jeffcott et al., 1986; Lawrence et al., 1994; Nielsen et al., 1997; Nielsen et al., 1998; Hoekstra et al., 1999; Bell et al., 2001; Hiney et al., 2004; Reichmann et al., 2004; Spooner et al., 2008). Weanlings exercised at a medium trot for up to 20 min, 5 d/wk tended to have an increased radiographic bone density of the medial cortices of the third

metacarpal bone (Raub et al., 1989). High intensity, short duration sprint exercise at 82 m/d, 5 d/wk increased bone mineral content in stalled weanlings compared to pasture-reared weanlings (Hiney et al., 2004). It has been suggested that training at low speeds will not necessarily stimulate appropriate bone modeling or remodeling in comparison to galloping (Nunamaker et al., 1990).

RBAE values have shown BMC in Quarter Horses in typical, industry race training to decrease from Day 0 to 56 and then increase up to 112 days. These findings follow the understood bone remodeling process initiated by exercise, starting with resorption activities and followed by bone formation (Nielsen et al., 1998). This aligns with the remodeling cycle timeline in humans where bone resorption takes approximately 2 to 4 wk, reversal can take up to 4 or 5 weeks (Hadjidakis and Androulakis, 2006), and bone formation takes approximately 4 to 6 mo to complete (Clarke, 2008).

Whole Body Vibration (WBV)

The use of vibration plates in human research in the last decade has gained substantial traction in hopes of finding a safe and effective treatment and prevention for osteoporosis and osteopenia from immobilization and disuse (Gusi et al., 2006). Whole body vibration (WBV) has been of great interest in the medical industry as a means to counteract bone loss from osteoporosis. The National Osteoporosis Foundation reports that approximately 54 million Americans have osteoporosis and low bone mass. This has attributed to 2 million broken bones and $19 billion in related costs every year (National Osteoporosis Foundation, 2015). Whole body vibration is a promising therapy to counteract osteoporosis, a prominent skeletal disease in humans.

Whole body vibration positively impacted bone density in young women (age 15-20 years) with low bone mineral density (BMD; (Gilsanz et al., 2006). Specifically, vibration at 30 Hertz, 10 minutes a day for 12 months resulted in increased bone density as reflected by the 2.1% increase in lumbar vertebrae trabecular BMD and the 3.4% increase in femoral midshaft cortical BMD. Many variations in WBV including magnitude, frequency, duration, and vibration direction (i.e. vertical, oscillatory) can influence results. One study of particular interest, as it relates to this research, looked specifically at the effects of vertical vibration in postmenopausal women. Whole body vibration for six months at 35-40Hertz resulted in a 0.93% increase from baseline in BMD of the hip (Verschueren et al., 2004). Slatkovska et al. (2010) performed a met-analysis of randomized controlled trials (RCTs) examining WBV. Eight RCTs including postmenopausal women (five RCTs), young adults (one RCT), and children and adolescents (two RCTs) were reviewed. The regimens were heterogeneous and study durations were relatively short. In postmenopausal women, WBV was found to significantly increase hip BMD versus controls, but not spine BMD or tibia trabecular BMD. In young adults, WBV did not increase spine or hipbone mineral content, or tibia trabecular BMD. In children and adolescents, WBV significantly increased spine trabecular BMD (Slatkovska et al., 2010).

Every day activity has been shown to be characterized more as low amplitude, high frequency on the bone (Fritton et al., 2000). Therefore, experiments testing WBV at a low amplitude, high frequency have been done in other species including sheep (Rubin et al., 2001a; Rubin et al., 2001b; C. Rubin et al., 2002; Clinton Rubin et al., 2002), rats (Flieger et al., 1998; Oxlund et al., 2003) and mice (Xie et al., 2006). In growing mice (8 weeks

old), vibration exposure of short durations of low magnitude and high frequency can inhibit trabecular bone resorption indicating a maintenance of current bone status (Xie et al., 2006). Collectively, these studies demonstrate that low amplitude, high frequency vibration can be effectively transmitted to the bone to stimulate an adaptive response.

Whole body vibration research in the horse has been limited to a minimal number of studies since 2013. Two studies focused on physiological effects of short-term vibration (Carstanjen et al., 2013; Buchner et al., 2017). Nowlin et al. (2018) tested the effects of both acute and prolonged vibration treatment on lameness. The remaining studies tested a more long-term exposure to WBV ranging from 28-120 d (Hulak et al., 2015; Halsberghe, 2017; Hyatt et al., 2017; Maher et al., 2017). The physiological responses measured varied widely across these studies.

Carstanjen et al. (2013) subjected seven adult horses to 10 min of WBV at 15-21 Hz. Clinical parameters, hematology, fibrinogen, lactate, IGF-I, GGT, creatinine, myeloperoxidase activity and bone biomarkers, osteocalcin and CTX-1, were measured. A significant decrease in cortisol and creatine kinase was observed. A drop in serum cortisol concentration could be indicative that stress levels did not increase as a result of exposure to vibration. However, other parameters, such as lactate and fibrinogen, remained unchanged. Most notable is that serum osteocalcin (serum marker of bone formation) and CTX-1 (serum marker of bone resorption) concentrations were not influenced by WBV. This is not surprising considering normal bone physiology. The low frequency of 15 to 21 Hz for a single session in this study was not enough to elicit a bone remodeling response (Carstanjen et al., 2013).

An identical vibration platform was used by Carstanjen et al. and Buchner et al. The vibration applied was both vertical and horizontal in direction. The entire vibration unit consisted of four independent platforms where each foot of the horse was positioned on one platform. Buchner et al. (2017) compared vibrated horses to those receiving a light warm up by longing. The study revealed that 10 min of vibration at 15 Hz (3 min), 22 Hz (4 min), and 25 Hz (3 min) did not stimulate major limb or back muscle groups in the same way a light warm up of longing did (Buchner et al., 2017).

Nowlin et al. (2018) evaluated the effect of both short-term and long-term vibration treatment on lameness. Six aged, Arabian horses were vibrated one time at 50 Hz for 30 min then re-evaluated for lameness. They then continued to be vibrated 5 d/wk for 3 wk and were re-evaluated again for lameness. Vibrated horses did not have significant changes in lameness score, stride length, lameness locator, or heart rate in comparison to controls at any point of re-evaluation.

The long-term studies conducted on WBV in the horse evaluated physiological responses related to bone and muscle and the therapeutic effects in chronic lameness. Hulak et al. (2015) compared WBV to light exercise in adult horses (mean age 17±4 yr) that were stalled. Vibrated horses stood on a vibration plate for 45 min at 50 Hz, 5 d/wk. In comparison, exercised horses were worked on a mechanical panel exerciser for 60 min, 6 times per week. After a 28 d treatment period, RBAE determined BMC to increase in both groups concluding that WBV maintained BMC in the same way light exercise would in stalled horses (Hulak et al., 2015). Although that study did not have a negative control and was performed in aged horses with mature skeletal structures, it took an important first step to assess WBV effects on the skeleton in horses.

This same group of researchers took another step towards evaluating WBV in 2017 by comparing horses exercised to those exercised and vibrated. Horses were stalled and radiographic bone aluminum equivalence values were calculated from radiographs taken at -28, 0, and 28 d. Stride length, heart rate, and serum biomarkers pyridinoline cross-links and osteocalcin were also evaluated. Results indicated no influence of WBV on RBAE values of any bone cortices or bone turnover biomarkers. However, there was a period effect of a decrease in RBAE lateral cortices and total bone density, which the author contributed to a likely effect of stalling. Stride length was also not different between the two groups. As suggested by anecdotal evidence, there was a significant difference in heart rate in horses vibrated in this study. Horses receiving vibration had a lower heart rate (-4.8 ± 2.83 bpm) than those that did not (3.0 ± 2.83 bpm) (Maher et al., 2017).

Hyatt et al. (2017) evaluated the effect of WBV on muscle metabolites on 20 stalled, yearling horses. Ten horses received vertical vibration of 50 Hz for 30 min, 5 d/wk for 120 d. All horses were allotted 30 min free turnout 5 d/wk throughout the duration of the study, with the treated group receiving vibration immediately following turnout and the control group being returned to their stalls. Serum was collected pre- and post-turnout or vibration on d 0, 30, 60, 90, and 120. Aspartate aminotransferase (AST) showed significant reduction across all serum collections in the control group. Gamma-glutamyltransferase values significantly declined between collections for both groups. There was also a treatment x day interaction for creatine kinase (CK). In conclusion, muscle metabolites were not significantly effected by WBV in this study (Hyatt et al., 2017).

Finally, Halsberghe et al. (2017) engaged in a pilot study to determine effects of WBV on chronic lameness in a single subject, repeated measures design. Some anecdotal

evidence has shown that WBV could be helpful for horses experiencing lameness. This study used horses with gradable lameness and a previous history of lameness to test WBV. They were subjected to WBV at a frequency of 40 Hz, amplitude of 0.8 mm for 30 min twice daily, 5 d/wk, for 60 d. A trend towards improvement in lameness was noted within the first 30 d. However, there was not a significant change in lameness after 30 or 60 d of WBV (Halsberghe, 2017).

To the authors' knowledge, there are no studies published on the influence of WBV on bone remodeling in pigs. A handful of studies have focused on stress response to vibration in livestock in an effort to better quantify and improve hauling welfare. It has been reported by measuring heartrate in pigs, that vibration sensitivity increases with acceleration magnitude (1 or 3 m/s^2) more than with frequency (2-18 Hz) (Perremans et al., 1998). Frequencies of vibration on a vehicle towing a trailer with dairy cattle measured at 85 km/h were 1.3, 5.1 and 12.6 Hz, with a secondary peak at 23 Hz along the vertical direction (Gebresenbet et al., 2011). It has been shown in sheep that low level, high frequency (20-50 Hz) mechanical strain in the form of an oscillatory platform can result in a 10.6% increase in bone mineral content (BMC) and trabecular number that is 8.3% higher over the period of a year of exposure (Clinton Rubin et al., 2002; Judex et al., 2003).

Methodological Approaches for Studying Bone

Many methodologies for assessing bone are available and vary in usefulness depending on the subject and information being solicited. Digital radiography is the primary diagnostic tool for veterinarians to use in diagnosing skeletal changes and injuries in livestock. It also has its usefulness in human diagnostics. Advances in digital

radiography have led to more defined parameters that can be assessed in comparison to conventional radiography. Radiographic absorptiometry (RA) and computed digital absorptiometry (CDA) have been shown to accurately assess bone mineral density (BMD) in humans (Compston et al., 1995; Bouxsein et al., 1997; Nolla et al., 2000) and horses (Vaccaro et al., 2012). Computed digital absorptiometry is similar to RA, using a single-energy X-ray source, an aluminum step-wedge, and a charge-coupled device (CCD) detector system to automatically compute bone mineral content (BMC, g) and bone mineral density (BMD, g/cm2)(Nolla et al., 2000). The aluminum step-wedge is a standard of various known thicknesses and densities that is included in each radiograph allowing for calibration from image to image. The grayscale at the region of interest (ROI) is determined by the brightness/darkness index as calibrated by the standard (Bowen et al., 2013). The advantage of CDA over RA is results can be obtained immediately (Bouxsein et al., 1997). Digital radiography has a distinct advantage over other methodologies by being inexpensive, portable, and easy to use in the field setting (Bowen et al., 2013).

Dual-energy x-ray absorptiometry (DXA) is considered the gold standard in diagnosing osteoporosis. It is a two-dimensional measurement that uses attenuation of x-ray beams with two different energies. Bone mineral content of a specific region can be obtained with DXA. Bone mineral density can be calculated using the obtained BMC and scanned area. It has also proven useful in capturing bone microarchitecture of the lumbar spine to determine a trabecular bone score (TBS), which is useful in addition to the BMD measurement (Silva et al., 2014). Dual-energy x-ray absorptiometry has disadvantages that include inability to distinguish cortical bone from trabecular bone and variability in BMD based on the size of the bone (Dhainaut et al., 2016).

Quantitative computed tomography (QCT) is similar to DXA in technique, however the detector rotates around the subject therefore allowing the construction of three-dimensional images from sequential two-dimensional sections (Dhainaut et al., 2016; Riggs, 2018) . Quantitative computed tomography also has the capabilities to determine a volumetric BMD (vBMD) independent of bone size and can differentiate between cortical and trabecular bone with high accuracy (Cornelissen et al., 1999; Boutroy et al., 2005; Donnelly, 2011). Bone geometry and density can be characterized as elements of bone risk fracture when using QCT. Bone mineral density only characterizes approximately 70 to 75% of bone strength (Leichter et al., 1982; Ammann and Rizzoli, 2003) and therefore other parameters of bone such as macro- and micro-architecture and tissue quality are important to measure which is possible with QCT (Burghardt et al., 2011). High resolution computed tomography or microCT is a higher resolution technique from QCT allowing for more refined measurements of microarchitecture and the three-dimensional reconstruction array is created directly (Feldkamp et al., 1989; Dhainaut et al., 2016). Although not readily accessible for most practical applications, QCT and microCT have proven very useful in detecting bone morphological changes to detect bone fracture in horses where other modalities failed (Beccati et al., 2017; Cresswell et al., 2018; Whitton et al., 2018).

Computed tomography (CT) has been used in the veterinary practice for nearly 30 yr, but with limited use in the equine due to the drawbacks with use. Equine scans require general anesthesia to place the horse in lateral recumbency to position the desired body part, usually the limb, within the annulus of the CT machine for imaging. Even under anesthesia there could be complications associated with the horse reacting violently and potentially damaging an expensive machine. Additionally, images are of a non-

weightbearing limb and the whole procedure can be costly (Riggs, 2018). In an attempt to overcome these challenges, a few groups have been working towards designing a CT machine that take images while the horse remains standing. These advancements hold great promise, but are still in the early stages of development and few are installed to date (Riggs, 2018).

Quantitative ultrasound (QUS) gives an indirect measure of bone density although not directly comparable to other densitometry methods (Dhainaut et al., 2016). It measures velocity and frequency-dependent attenuation. Quantitative ultrasound has found a niche measuring peripheral skeletal sites, such as the metacarpal bone, and has gained popularity due to its portability and cost savings in comparison to other technologies such as DXA and QCT (Krieg et al., 2008). Quantitative ultrasound has found usefulness in bone assessment in the equine industry due to its convenience and non-radiation methods, making it useful in onsite and clinical settings (Lepage et al., 2001).

Some comparative studies have been conducted to validate use of imaging techniques to assess bone. Mean gray value (MGV) from radiographs of bovine metatarsal bones and equine femurs were compared to bone mineral density measurements taken with DXA. The MGV of conventional and digital radiography was highly correlated with BMD measurements from DXA (0.910 and 0.937, respectively) (Vaccaro et al., 2012). It has been noted that conventional radiographs can be limited in their usefulness to diagnose fractures in the horse, often failing to detect small fractures, especially those that are fairly recent (Kawcak et al., 1995). Comparative diagnosis between radiographs and CT of osteomyelitis in foals resulted in an 37% underestimation of the area of lesion by

radiography. Radiography projections were also 2.5-fold more variable in the measurement area compared with CT (Lean et al., 2018).

SUMMARY

Despite the prevalence of anecdotal claims that WBV therapy improves physiological functions or increases bone density, conclusive evidence does not exist to substantiate these claims especially in the young, growing animal. This dissertation research project was designed to assess the purportedly positive effects of WBV on bone metabolism, microarchitecture, and mineralization in growing pigs and yearling horses. To investigate the effects of WBV and Ca and P levels on bone quality and turnover, digital radiographs with an aluminum step wedge determined bone mineral content by radiographic bone aluminum equivalency (RBAE) and serum biomarkers of bone formation (osteocalcin, OC) and bone resorption (carboxy-terminal collagen crosslinks, CTX-I) were measured in pigs and horses.

CHAPTER III

RESPONSE OF BONE MICROARCHITECTURE AND BIOMARKERS OF BONE METABOLISM TO WHOLE BODY VIBRATION AND DIETARY CALCIUM AND PHOSPHORUS IN GROWING PIGS

INTRODUCTION

Skeletal integrity is vital for economic sustainability of swine operations. Pigs in the production cycle must remain sound or be culled. Many factors contribute to bone quality and strength for soundness, with adequate mineral intake of high priority. Calcium and phosphorus are two important diet considerations for adequate skeletal growth and maintenance. Dietary Ca and P are bound together in the bone at a constant ratio of 2.2:1 and the skeleton is a reserve for both Ca and P (Crenshaw et al., 2001a). A balance must be achieved to avoid excess or deficiency when feeding minerals such as Ca and P for skeletal development (Doige et al., 1975). Excess mineral intake of P has resulted in decreased bone growth, skeletal material, and structural properties and decreased bone strength in growing rats despite adequate Ca intake (Huttunen et al., 2006). High levels of Ca and P intake in growing pigs did not increase mineral accretion in bone and decreased bone resorption, therefore Fernández (1995) concluded optimal mineral supply for normal bone development and turnover is more complicated than simply increasing mineral intake. Additionally, Wu et al. (2018) demonstrated that excess dietary Ca negatively affected growth performance and percentage bone ash of nursery pigs when diets were deficient in P. Finally, increasing attention has been placed on the environmental impact of feeding excess P and therefore identifying adequate feeding rates is important (Poulsen, 2000). Diets deficient in Ca and P can predispose pigs to bone fractures resulting in economic

loss. Bone mineral density and content was decreased in growing pigs fed diets deficient in P (Liesegang et al., 2002; Gonzalo et al., 2018).

Other contributing factors to bone quality and quantity include strain and concussion from activities such as exercise. The body performs many activities that increase load and concussion to the skeleton and this includes vibration. Vibration can be experienced in many forms including through exercise. Perhaps one of the most common is vehicle transportation, or in the animal's case, trailer transportation. Frequencies of vibration on a vehicle towing a trailer with dairy cattle measured at 85 km/h were 1.3, 5.1 and 12.6 Hz, with a secondary peak at 23 Hz along the vertical direction (Gebresenbet et al., 2011). Vibration from daily activities can be mimicked in a controlled manner by the use of whole body vibration, which is a therapy that exposes an individual to mechanical oscillations typically by standing on a vibrating platform. Some studies have shown WBV to have an osteogenic effect and increase bone quality and quantity in humans and rodents (Oxlund et al., 2003; Verschueren et al., 2004; Gilsanz et al., 2006; Xie et al., 2006; Pasqualini et al., 2013; Vanleene and Shefelbine, 2013). It has been shown in sheep that low level, high frequency (20-50 Hz) mechanical strain in the form of a oscillatory platform can result in a 10.6% increase in bone mineral content (BMC) and trabecular number that is 8.3% higher over the period of a year of exposure (Clinton Rubin et al., 2002; Judex et al., 2003). In growing mice (8 weeks old), vibration exposure of short durations of low magnitude and high frequency can inhibit trabecular bone resorption indicating a maintenance of current bone status (Xie et al., 2006). The use of vibration plates in human research in the last decade has gained substantial traction in hopes of finding a safe and effective treatment and prevention for osteoporosis.

To the author's knowledge, no studies have been performed in swine to evaluate bone quality and quantity as a result of whole body vibration therapy with the use of a vibration platform. To better understand this therapy's influence on bone in humans and horses, a preliminary study in pigs was performed and utilized additional bone quality and quantity testing techniques that can be performed *ex vivo* including micro computed tomography.

Pigs have been extensively used as a reliable biological model for humans in biomedical research due to their distinct similarities in anatomy, physiology, metabolism and nutrition (Bustad, 1966; Douglas, 1972; Pong, 1978; Miller and Ullrey, 1987; Swindle et al., 2012). There are also some important similarities in bone composition between humans and the pig (Dickerson, 1962). The trial was conducted using growing pigs as a biological model to: **a)** determine effects of whole body vibration stimulus on changes in bone density and composition and **b)** determine changes to bone composition due to low and adequate dietary levels of Ca and P.

This dissertation research examined the purported positive effects of whole body vibration (WBV) on bone parameters (turnover, bone mass and microarchitecture) using the pig model. Although evidence exists supporting the idea that WBV therapy improves physiological functions or increases bone density, very little conclusive evidence exists to substantiate these claims.

MATERIALS AND METHODS

Animals

A total of 26 Yorkshire cross pigs (initial average BW 40.2 kg) were used in this study. Pigs belonged to Texas A&M University and were individually housed in pens (1.32 x 0.71 m) in the Mineral Studies room of the Nutrition and Physiology Center (NPC) at the Animal Science, Teaching, Research, and Education Center (ASTREC). Pigs were acclimated to diet and housing seven days prior to start of the trial. Ambient temperature of the room was maintained between 70-78°F. Use of animals was approved by the Texas A&M University Institutional Agricultural Animal Care and Use Committee using guidelines set forth by the Federation of Animal Science Societies (2015).

Treatments

Pigs were blocked by gender and group and balanced by initial weight, then assigned to one of four treatments. Treatments were arranged in a 2 × 2 factorial with factors consisting of dietary Ca and P concentration (adequate vs low) and WBV (no vibration or subject to vibration (Table 1).

Table 1. Treatment group for pigs (n = 26).

TRT Group	Diet-NRC (Ca-P)	WBV Therapy	n
I	Adequate	No	n = 6
II	Low-Ca, P	No	n = 7
III	Adequate	Yes	n = 7
IV	Low-Ca, P	Yes	n = 6

Pigs receiving vibration treatment were backed out of their individual pens and walked to the vibration plate (Equivibe, Lincoln, NE), which is a stationary platform 4 inches off the ground. Pigs were contained on the plate with a freestanding pen and vibrated for 30 min per day, 3 days per week at a high frequency of 50 hertz and a low magnitude of 1-2mm. Two pigs separated by a panel received whole body vibration at the same time for 30 minutes and then were returned to their pen. Pigs alternated between which side they stood on the vibration plate each time they received the vibration treatment. Control pigs were also backed out of their individual pens and walked to the vibration plate and then returned to their pens with no vibration treatment.

For dietary treatment, the adequate diet was formulated to meet all nutritional needs according the NRC recommendations for growing pigs (NRC, 2012). Low-Ca, P diet was formulated to be 0.9 g/kg less than the recommended levels of available P and Ca, but adequate in all other nutrients. Samples diets were collected by randomly selecting samples of feed at d 30 and 60. Feed samples were submitted to a commercial laboratory for detailed analysis (Table 2) (Cumberland Valley Analytical Services, Hagerstown, MD). Feed at d 60 was based on corn, soybean meal, and monocalcium P analyzed for calcium and phosphorus and used for diet formulation. Pigs had ad libitum access to feed and water throughout the trial. Feeders were monitored daily and feed was weighed, recorded, and added twice daily, as needed. Refusals were collected before each feeding, then dried and weighed to subtract from intakes recorded.

Table 2. Diet composition (as-fed basis)

Ingredient	Day 0 to 30		Day 30 to 60	
	Adequate	Low Ca, P	Adequate	Low Ca, P
Corn	75.54	76.4	80.89	81.72
Soybean meal (48% CP)	21.77	21.71	16.83	16.77
Monocalcium P	0.90	0.20	0.78	0.10
Limestone	0.83	0.73	0.60	0.50
Salt	0.35	0.35	0.35	0.35
L-Lys HCl	0.28	0.28	0.25	0.25
DL-Met	0.03	0.03	0.01	0.01
L-Thr	0.06	0.06	0.05	0.05
Vitamin and trace mineral premix[1]	0.25	0.25	0.25	0.25
Calculated composition				
CP, %	16.9	17	15	15
ME, Kcal/kg	3,291	3,318	3,307	3,333
Ca, %	0.54	0.38	0.42	0.27
P,%	0.54	0.40	0.50	0.35
STTD of P, %	0.31	0.18	0.28	0.15
Feed Analysis Composition				
Ca, %	0.67	0.44	--[2]	--[2]
P, %	0.52	0.36	--[2]	--[2]

[1]Provided per kilogram of premix, 3,520,000 IU vitamin A, 661,380 IU vitamin D_3, 1,763,680 IU vitamin E, 441 mg menadione, 2,204 mg riboflavin, 8,818 mg pantothenic acid, 13,228 mg niacin, 13 mg vitamin B12, 8,818 mg pantothenic acid, 88.2 mg biotin, 441 mg thiamine, 441 mg folic acid, 882 pyridoxine, 8.9 g Mn, 50 g Fe, 50 g Zn , 6.05 g Cu, 240 mg I, and 120 mg Se

[2]Corn, soybean meal, and monocalcium P were analyzed for calcium and phosphorus. Analyzed values were used for diet formulation.

Physical Measurements

On Day 0, 30, and 60 of the trial, individual body weight was measured by walking each pig to a calibrated standard livestock scale. Feed intake was measured and used with weight to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F).

Direct Measurements of Bone - Digital Radiographic Analysis

Radiographs of the left, third metacarpal bone and aluminum step wedge were taken on Day 0, 30, and 60 using a Portable Veterinary X-ray unit (MinXray, Inc, Northbrook, IL). A dorsal-palmar view was taken at a focal distance of 26 cm and exposure of 76 kVp and 0.06 ms. Images were produced by snaring the pigs in an elevated crate and then draping the left front leg over a 10x10 inch cassette over the edge of the crate. An aluminum step wedge penetrometer of 11 steps ranging from 5 mm to 35 mm in 3mm increments was attached to each radiographic cassette on the same side. The aluminum step wedge was used to standardize readings and determine RBAE values using software validated by O'Connor-Robison and Nielsen (2013).

Original digital radiograph files were converted to 16-bit TIFF picture files using the open source software ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA.). Quantity One 1-D analysis software (BioRad, Hercules, CA) was then used to analyze TIFF files. Images were rotated to orient the third metacarpal bone in a vertical position and enlarged to life size, using the penetrometer as a guide. The Volume Rect Tool was used to draw a box around the area of interest. The same sized box was copied and pasted on each step of the AL step wedge (11 steps total) and on the cortical bone. The exact size of box was confirmed by the identical area provided by the Volume Analysis Report. Each individual bone was measured from the proximal end to the distal end and two boxes were placed on the cortical bone at the half way point on the diaphysis. One box was placed to encompass the medial aspect and the other placed to encompass the lateral aspect of the cortical bone. The Volume Analysis Report was used to obtain data from these boxes on the following parameter:

Max Value (INT) - The value of the highest intensity pixel in the volume

Values for each parameter were obtained from the boxes for each step of the AL step wedge and were used to develop a best-fit linear equation to predict the values of the medial and lateral boxes of the cortical bone.

Radiographs were also taken of the excised metacarpal bones post-harvest. Six or seven bones were placed from left to right in the same orientation (proximal/distal) on each cassette with the AL step wedge and radiographed and analyzed in the same manner as described above.

Direct Measurements of Bone - Micro Computed Tomography (CT)

After harvest, bones were fixed in 10% neutral buffered formalin, covered, and sealed and stored at room temperature until imaging. High resolution micro-CT (μCT 50 (Scanco Medical, Brüttisellen, Switzerland) was used to qualitatively and quantitatively analyze specimens (Hildebrand and Ruegsegger, 1997a; Hildebrand and Ruegsegger, 1997b). Samples were scanned in a μCT 50 (Scanco Medical, Brüttisellen, Switzerland) with parameters: 70 kVp, 76 μA, 0.5 Al Filter, 900 ms integration time, and 20 μm voxel size calibrated to 5 known densities (mg/cm³) of hydroxyapatite according to approval guidelines (Bouxsein et al., 2010). The third metacarpal metaphysis was analyzed for trabecular architecture and volumetric bone mineral density (Fig. 3). Fractional bone volume (bone volume/total volume; BV/TV) and architectural properties of trabecular bone, thickness, number, and spacing were calculated for each excised left, third metacarpal bone. The following parameters were calculated:

TV (mm^3)-total volume

BV (mm^3)-bone volume

BV/TV (%)-relative bone volume

Tb.N (1/mm)-trabecular number

Tb.Th (mm)-trabecular thickness

Tb.Sp (mm)-trabecular separation = marrow thickness

Conn.D. (1/mm^3)-connectivity density, normed by TV

SMI (0 for parallel plates, 3 for cylindrical rods)-structure model index

vBMD (g/cm^3)

DA (1= isotropic, › 1 anisotropic by definition DA = length of longest divided by shortest H-vector)-degree of anisotropy

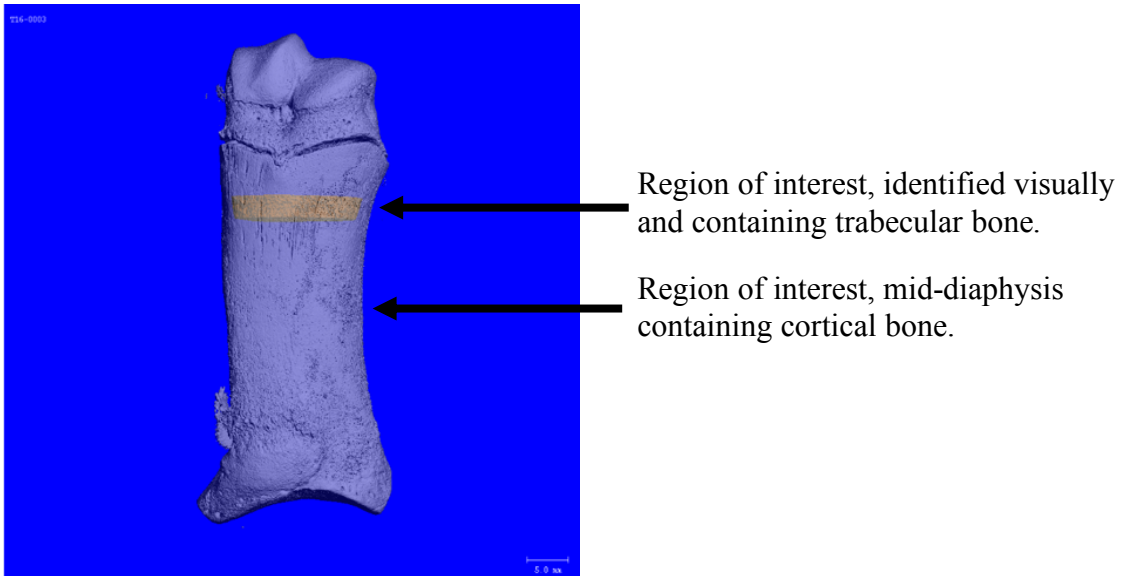

Figure 3. Region of interest for analysis of trabecular and cortical bone by microCT of pig third metacarpal bone.

The CT images of the mid-diaphysis of the third metacarpal for cortical bone analysis were segmented into bone and marrow regions by applying a visually chosen, fixed threshold for all samples, after smoothing the image with a three-dimensional Gaussian low-pass filter. The outer contour of the bone was found automatically with the

built-in Scanco iterative contouring tool. Total area was calculated by counting all voxels within the contour, bone area by counting all voxels that were segmented as bone, and marrow area was calculated as total area − bone area. This calculation was performed on all 20 slices (1 slice = 12.5 μm), using the average for the final calculation. The outer and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone surface (BS) of the 20 slices, and cortical thickness was calculated (Bagi et al., 2006). The following parameters were calculated:

Cort. CSA (mm)-cortical cross-sectional area

Cort. Th (mm)-cortical thickness

Endo. Perimeter (mm)-endosteal perimeter

Bone Biomarkers

Blood was collected on Day 0, 30, and 60 via jugular venipuncture using a 3.81 cm, 20-gauge needle. A 10mL purple-top vacutainer (EDTA) and a 10mL red-top vacutainer (no additive) were collected. Red-top tubes were allowed to clot at room temperature for 20 min, then transferred to an ice chest before being centrifuged at room temperature (<6 hr elapsed from time of collection to harvest). Purple-top tubes were immediately transferred to an ice chest before being centrifuged at 2000 x g at 4°C for 20 minutes. Serum and plasma was collected and stored at -80°C until analysis. Serum was analyzed for biomarkers to measure the rate of bone turnover (remodeling). Serum concentration of osteocalcin was determined via Rat-MID osteocalcin enzyme immunoassay (EIA) kit (Immunodiagnostic Systems Holdings PLC, Gaithersburg, Maryland) as indicator of osteoblast activity indicating bone osteogenesis (formation). Serum concentration of carboxy-terminal collagen crosslinks (CTX-1) was determined via human C-telopeptide of

collagen (CTX) ELISA kit (Immunodiagnostic Systems Holdings PLC, Gaithersburg, Maryland) as an indicator of bone resorption. Kits were validated by dilutional parallelism and intra and inter assay variability. Evaluated samples were assayed within the linear range.

Post-Harvest Carcass Measurements

Pigs were transported on Day 60 to K & C Meat Processing (Navasota, TX) for harvest. Immediately following harvest, the left leg was removed from the carcass at the knee and transported on ice to the Endocrine Physiology Laboratory within the Department of Animal Science in Kleberg Animal and Food Sciences Center. The third metacarpal bone of each front limb was carefully dissected and cleaned of any tendons and muscles. Bones were then placed in containers filled with 10% neutral buffered formalin, covered, and sealed and stored at room temperature. Carcass measurements were taken one day post-harvest. Measurements included hot carcass weight. Carcass weight ranged from 60.9 kg to 85 kg (mean = 73.3 kg).

Statistical Analysis

Data was analyzed as a randomized complete block design using the MIXED procedure of SAS (SAS Institute INC., Cary, NC) with pig as the experimental unit. Ca and P level, vibration treatment, and Ca and P level × vibration therapy served as fixed effects. Least squares means were calculated and tested for significance using the pdiff procedure of SAS (SAS Institute INC., Cary, NC). For radiographic AI-step wedge, ultrasound, and blood measurements the statistical structure was the same except day of measurement and all interactions served as fixed effects in addition to treatment. Day was analyzed using linear and quadratic polynomials for equally spaced treatments. Day of

measurement also served as the repeated measure with animal as the subject. Statistical significance was determined at $P < 0.05$ and $P < 0.10$ was considered a trend.

RESULTS

Growth Performance and Carcass Characteristics

There were no whole body vibration (WBV) × diet interactions or main effects of diet or vibration ($P > 0.05$) on growth performance of finishing pigs (Table 3). There were no WBV × diet interactions ($P > 0.05$) for hot carcass weight (HCW). Pigs vibrated had increased ($P = 0.041$) HCW compared to the controls. Pigs fed diets with increased concentration of Ca and P had increased ($P < 0.05$) HCW compared to those fed diets with low concentrations of Ca and P.

Table 3. Main effects of whole body vibration (WBV) and Ca and P levels on growth performance and carcass characteristics of finishing pigs[1]

	Diet			WBV			*P*-Value[2]	
	Adequate	Low-Ca, P	SEM	No	Yes	SEM	WBV	Diet
BW, kg								
d 30	68.27	67.68	4.31	68.09	67.86	4.31	0.793	0.558
d 60	93.77	91.77	7.73	92.77	92.77	7.72	0.986	0.264
d 0 to 60								
ADG, kg	0.950	0.914	0.125	0.932	0.932	0.125	0.996	0.252
ADFI, kg	2.57	2.57	0.498	2.60	2.54	0.498	0.429	0.928
G:F	0.37	0.36	0.148	0.36	0.37	0.148	0.377	0.152
HCW, kg	73.86	69.86	3.70	69.78	73.80	3.704	0.041	0.050

[1]A total of 26 finishing pigs (average initial BW = 40.2kg) were used in a 60-d trial. Pigs were individually housed, n = 6 (adequate diet, no vibration) n = 7 (low Ca, P, no vibration) n = 7 (adequate diet, vibrated) n = 6 (low Ca, P, vibrated).

[2]No WBV x diet interaction was observed ($P > 0.05$).

Radiographic Bone Aluminum Equivalency (RBAE)

There were no WBV × diet × day or two-way interactions ($P > 0.14$) on max RBAE values for the medial or lateral cortices of the third metacarpal bone in finishing pigs (Table 4). There was no evidence for difference between vibrated pigs or control pigs on RBAE max values for the medial or lateral cortices. Pigs fed a diet with adequate concentrations of Ca and P tended to have increased RBAE max values for the medial and lateral cortices compared to those fed a diet with deficient concentrations of Ca and P. Mean RBAE max values for medial cortices increased (linear, $P = 0.028$) in pigs from d 0 to 60. Mean RBAE max values for the lateral cortices had a marginally significant increase (quadratic, $P = 0.084$) from d 0 to 60.

Table 4. Main effects of whole body vibration (WBV) and Ca and P levels on radiographic bone aluminum equivalence (RBAE) max values of medial and lateral cortices of the third metacarpal bone in finishing pigs[1]

	Diet		WBV			*P*-value[2]							
	Adequate	Low-Ca, P	Yes	No	SEM	WBV × Day, Linear	WBV × Day, Quadratic	Diet × Day, Linear	Diet × Day, Quadratic	WBV	Diet	Day, Linear	Day, Quadratic
Medial Cortices[3,4]													
d 0	9.31	8.74	9.22	8.83	1.063	0.796	0.140	0.513	0.311	0.713	0.102	0.028	0.464
d 30	9.89	9.79	9.53	10.15									
d 60	10.69	9.50	10.41	9.77									
Lateral Cortices[3,4]													
d 0	9.55	9.00	9.56	8.99	1.358	0.793	0.237	0.488	0.614	0.493	0.101	0.453	0.084
d 30	10.63	10.15	10.16	10.61									
d 60	10.47	9.04	10.20	9.30									

[1]A total of 26 finishing pigs (average initial BW = 40.2kg) were used in a 60-d trial.

[2]No Vibration x Diet x Day interaction was observed ($P > 0.05$).

[3]Initial Day 0 RBAE max values used as a covariate.

[4]RBAE max values in mm Al.

Micro-Computed Tomography (CT) -Trabecular and Cortical Bone

There were no WBV × diet interactions ($P > 0.12$) for architectural parameters of BV/TV, Conn D., SMI, Tb.Th, Tb. Sp, vBMD, or TRI-DA (Table 4). A WBV × diet interaction ($P = 0.025$) for Tb.N was observed. Pigs not receiving vibration treatment and fed either diet had similar Tb.N for the third metacarpal bone. Conversely, pigs that received vibration and were fed the adequate diet had increased Tb.N compared to those vibrated and fed a low Ca and P diet. Pigs receiving vibration treatment had increased ($P = 0.003$) Tb.Sp values compared to those not vibrated. All other microCT parameters measured were not significantly different between pigs vibrated and control animals. Diet was not a significant contributor to any differences between microCT parameters measured between pigs fed an adequate diet and pigs fed a low Ca and P diet.

There were no WBV × diet interactions ($P > 0.355$) for Cort. CSA and Endo. Perimeter, but there was a tendency for an interaction ($P = 0.085$) for Cort. Th (Table 7). Pigs that were not vibrated tended to have higher Cort. Th values when fed the adequate Ca, P diet. There was no effect ($P > 0.185$) of vibration or diet for all cortical bone parameters between treated pigs and controls.

Table 5. Interactive effects of whole body vibration (WBV) and Ca and P levels on microCT of trabecular bone of third metacarpal in finishing pigs[1]

	No Vibration		WBV			*P*-value[2]		
	Adequate Diet	Low-Ca, P	Adequate Diet	Low-Ca, P	SEM	WBV	Diet	WBV × Diet
BV/TV (%)	0.287	0.274	0.276	0.237	0.018	0.150	0.124	0.410
Tb.N (1/mm)	2.05	2.136	1.975	1.721	0.082	0.002	0.247	0.025
Tb.Th (mm)	0.165	0.162	0.165	0.160	0.007	0.898	0.590	0.897
Tb.Sp (mm)	0.481	0.459	0.534	0.605	0.030	0.003	0.409	0.120
Conn.D. ($1/mm^3$)	12.79	12.02	13.02	11.22	1.110	0.793	0.245	0.636
SMI	0.527	0.455	0.555	0.729	0.117	0.183	0.646	0.273
vBMD	677.83	668.13	679.42	676.46	6.710	0.453	0.340	0.608
TRI-DA	1.90	1.94	1.88	1.87	0.043	0.332	0.695	0.600

[1]A total of 26 finishing pigs (average initial BW = 40.2kg) were used in a 60-d trial. Pigs were individually housed, n = 6 (adequate diet, no vibration) n = 7 (low Ca, P, no vibration) n = 7 (adequate diet, vibrated) n = 6 (low Ca, P, vibrated).
[2]No WBV x diet interaction was observed ($P > 0.05$).

Table 6. Main effects of whole body vibration (WBV) and Ca and P levels on microCT parameters of trabecular bone of third metacarpal in finishing pigs[1]

	Diet			WBV			*P*-value	
	Adequate	Low-Ca, P	SEM	No	Yes	SEM	WBV	Diet
BV/TV (%)	0.281	0.256	0.013	0.281	0.257	0.013	0.150	0.124
Tb.N (1/mm)	2.01	1.93	0.062	2.09	1.850	0.062	0.002	0.247
Tb.Th (mm)	0.165	0.161	0.005	0.163	0.162	0.005	0.898	0.59
Tb.Sp (mm)	0.508	0.532	0.020	0.47	0.57	0.020	0.003	0.409
Conn.D. ($1/mm^3$)	12.91	11.62	0.758	12.41	12.12	0.758	0.793	0.245
SMI	0.541	0.592	0.082	0.491	0.642	0.083	0.183	0.646
vBMD	678.62	672.3	4.57	672.98	677.94	4.57	0.453	0.340
TRI-DA	1.89	1.90	0.031	1.92	1.88	0.031	0.332	0.695

[1]A total of 26 finishing pigs (average initial BW = 40.2kg) were used in a 60-d trial. Pigs were individually housed, n = 6 (adequate diet, no vibration) n = 7 (low Ca, P, no vibration) n = 7 (adequate diet, vibrated) n = 6 (low Ca, P, vibrated).

Table 7. Interactive effects of whole body vibration (WBV) and Ca and P levels on microCT parameters of cortical bone of third metacarpal in finishing pigs[1]

	No WBV		WBV			*P*-value		
	Adequate Diet	Low-Ca, P	Adequate Diet	Low-Ca, P	SEM	WBV	Diet	WBV × Diet
Cort. CSA (mm)	2.378	2.495	2.523	2.484	0.09	0.424	0.636	0.355
Cort. Th (mm)	0.170	0.146	0.150	0.153	0.01	0.444	0.185	0.085
Endo. Perimeter (mm)	0.382	0.368	0.376	0.372	0.02	0.937	0.550	0.730

[1]A total of 26 finishing pigs (average initial BW = 40.2kg) were used in a 60-d trial. Pigs were individually housed, n = 6 (adequate diet, no vibration) n = 7 (low Ca, P, no vibration) n = 7 (adequate diet, vibrated) n = 6 (low Ca, P, vibrated).

Serum Bone Biomarkers - Osteocalcin (OC) and Carboxy-Terminal Collagen Crosslinks (CTX-1)

There were no WBV × diet × day interactions ($P > 0.05$) on bone turnover biomarkers osteocalcin and carboxy-terminal collagen crosslinks (CTX-1; Table 8). There was a tendency (linear, $P = 0.067$) for interactive effects between vibration and day for CTX-1 concentrations. Pigs receiving vibration treatment had decreased concentrations at d 30 and 60 compared to pigs not vibrated (Figure 4). There was no difference in CTX-1 concentrations between pigs fed an adequate diet and pigs fed a low Ca and P diet.

Interactive effects were observed between diet and day (linear, $P < 0.0001$) for osteocalcin concentration. Pigs fed an adequate diet had decreased concentration of osteocalcin from d 0 to 30, and then increased concentrations from d 30 to 60. Therefore, osteocalcin concentrations in pigs fed an adequate diet in Ca and P were similar at d 0 and 60. Pigs fed a low Ca and P diet had increased osteocalcin concentrations from d 0 to 60 (Figure 5).

Table 8. Main effects of whole body vibration (WBV) and Ca and P levels on bone biomarkers of finishing pigs[1]

	Diet		WBV			*P*-value[2]							
	Adequate	Low-Ca, P	Yes	No	SEM	WBV × Day, Linear	WBV × Day, Quadratic	Diet × Day, Linear	Diet × Day, Quadratic	WBV	Diet	Day, Linear	Day, Quadratic
CTX-1[3,4]													
d 0	0.160	0.160	0.163	0.156	0.025	0.067	0.665	0.946	0.120	0.044	0.156	<0.0001	0.019
d 30	0.160	0.220	0.169	0.211									
d 60	0.301	0.310	0.273	0.336									
Osteocalcin[3,5]													
d 0	2471.39	2499.30	2484.64	2486.04	201.28	0.978	0.504	<.0001	0.431	0.638	<.0001	<.0001	0.001
d 30	1980.02	2911.04	2523.72	2367.34									
d 60	2449.81	3920.34	3188.17	3181.98									

[1]A total of 26 finishing pigs (average initial BW = 40.2kg) were used in a 60-d trial. Pigs were individually housed, n = 6 (adequate diet, no vibration) n = 7 (low Ca, P, no vibration) n = 7 (adequate diet, vibrated) n = 6 (low Ca, P, vibrated).

[2]No Vibration x Diet x Day interaction was observed ($P > 0.05$).

[3]Initial Day 0 biomarker values used as a covariate.

[4]Bone resorption biomarker (ng/mL).

[5]Bone formation biomarker (ng/mL).

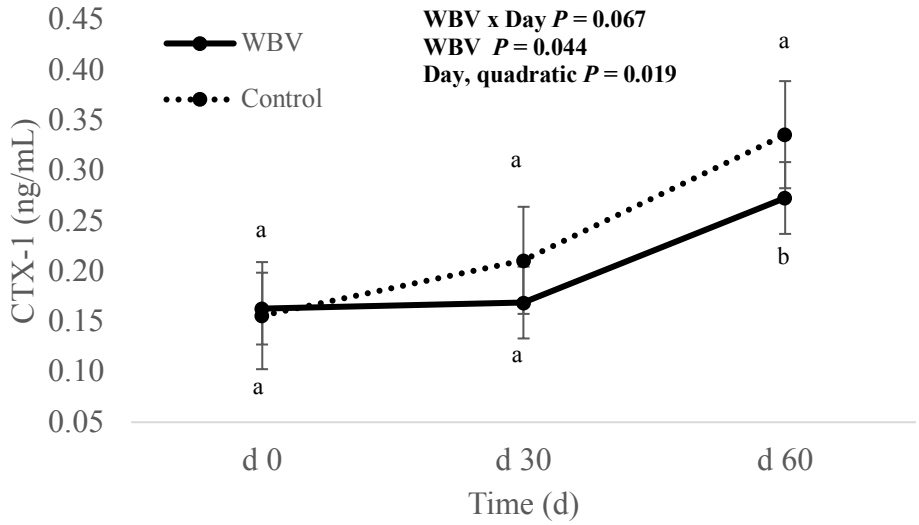

Figure 4. Changes in serum concentrations of CTX-1 (ng/mL) over time (d) for pigs receiving WBV (treatment) and those not receiving vibration (Control). Means differ at a time with no letters in common $P < 0.05$.

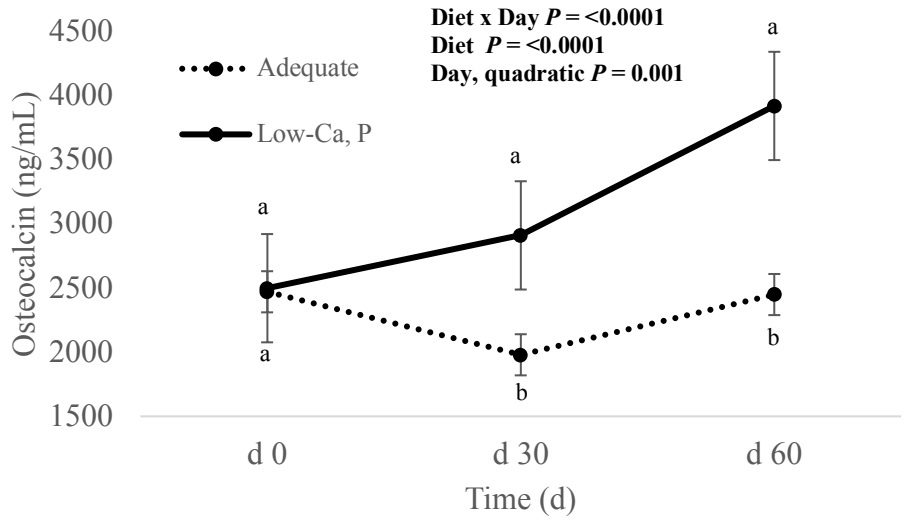

Figure 5. Changes in serum concentrations of osteocalcin (ng/mL) over time (d) for pigs fed a diet low in Ca and P (Treatment) and pigs fed a diet adequate in Ca and P (Control). Means differ at a time with no letters in common $P < 0.05$.

DISCUSSION

The primary objective of this study was to determine the effects of whole body vibration (WBV) and dietary levels of Ca and P on bone mass and bone microarchitecture in pigs. We hypothesized that WBV would have an anabolic effect on bone and therefore improve bone quality and quantity. Bone quality is the totality of properties that make bone resistant to fracture, and includes microarchitecture, accumulation of microscopic damage, quality of collagen, mineral crystal size, and bone turnover (Bouxsein, 2003; Fyhrie, 2005). Robust bones defy failure while fragile bones are more prone to fail (Fritton J and Schaffler M, 2008).

We also hypothesized that decreased levels of Ca and P in the diet would have increase bone resorption leading to deleterious effects on the bone. Deficient dietary levels of Ca and P have been shown to increase bone resorption, which can predispose pigs to lameness, fracture, and potential economic loss for the producer (Jensen et al., 2012). Calcium and P are two critical macro minerals for adequate skeletal growth and maintenance (Crenshaw et al., 2001b). Complex homeostatic mechanisms maintain blood Ca to mitigate hypocalcemia and hypercalcemia. Bone serves as a mineral depot for the body and is used to restore Ca concentrations in the blood, and in most cases can restore homeostatic conditions (Clarke, 2008). Results from this study confirm the expected bone changes due to a sustained low Ca and P diet. During hypocalcemia, immediate actions of the parathyroid glands, kidneys, and gastrointestinal tract bring blood Ca concentrations back to normal homeostatic conditions. If needed, longer term actions to restore serum Ca are taken by PTH and calcitriol to stimulate osteoclast activity which release Ca from bone. Increased osteoclastic activity leads to increased bone resorption (Littledike and

Goff, 1987). Most bone resorption markers are indicators of collagen breakdown during osteoclast activity. Carboxy-terminal cross-linked telopeptides of type I collagen (CTX-I) is a widely used bone resorption marker that has relatively high sensitivity and specificity for the degradation of type I collagen. Mean concentrations for CTX-I in this study progressively increased over the 60 d period in pigs fed the low Ca, P diet, which would be expected with increased resorption activity to maintain blood Ca levels. Findings by Eklou-Kalonji et al., (1999) closely aligned with this study when testing a similar dietary Ca concentration. Deficient bone mineralization combined with an increased bone resorption was observed in growing pigs fed for 32 d on diets low (0.38%) and very low (0.11%) in Ca (Eklou-Kalonji et al., 1999). Additionally, pigs fed low P levels (4.1 g/kg DM) also resulted in increased serum CTX-I concentrations in a study by Sørensen et al. (2018). The scope of this study did not permit measurement of other important indicators of Ca and P status, such as blood Ca and P, along with PTH and calcitriol. However, it has been demonstrated that changes in blood Ca may not be detectible except in cases of extreme Ca deficiencies in the diet (Eklou-Kalonji et al., 1999). An increase in plasma PTH would be expected during hypocalcemia to increase Ca retention in the kidneys, conversion of 25-hydroxy vitamin D into calcitriol, and bone resorption. Consequently increases in plasma calcitriol would also be observed in attempt to return Ca to homeostatic conditions (Eklou-Kalonji et al., 1999).

Bone resorption is coupled with bone formation (Parfitt, 1982). Osteoblasts secrete new bone matrix (osteoid) that is subsequently mineralized to fill in the resorptive cavity made by osteoclasts (Clarke, 2008). Bone formation biomarker OC can be measured to determine the level of osteoblastic activity (Brown et al., 1984; Charles et al., 1992;

Weaver et al., 1997). Osteocalcin is not released during bone resorption and therefore measured levels are interpreted as osteoblastic activity during bone formation (Price et al., 1981). Serum OC concentrations were greater in pigs fed low Ca, P diet in comparison to those fed the control. This could be attributed to the coupled action of bone resorption and formation, which has been observed by others (Eklou-Kalonji et al., 1999; Shaw et al., 2006).

Although the bone turnover markers are demonstrative of a resorptive process, the dietary decrease in the Ca, P did not produce a change in micro computed tomography (micoCT) parameters of the excised bone in either trabecular or cortical bone. Means for BV/TV and Tb.N were lower in pigs fed the low Ca, P diet, while Tb.Sp was higher, suggestive of a bone resorption response, although not statistically significant. There was a tendency for both medial and lateral cortices radiographic bone aluminum equivalency (RBAE) values to be lower in pigs fed the low Ca, P diet indicating a decrease in bone mineral content, likely resulting from resorption activities.

Bone turnover changes in response to WBV are mixed across human and rodent studies. Some literature has indicated bone-strengthening effects from WBV (Flieger et al., 1998; Rubin et al., 2001b; Oxlund et al., 2003; Verschueren et al., 2004; Gilsanz et al., 2006; Gusi et al., 2006; Xie et al., 2006) while others have shown no effect (Torvinen et al., 2003). In this study, WBV treatment did elicit a response change in trabecular bone parameters TbN. and Tb.Sp. Pigs that were vibrated had lower Tb.N values and higher Tb.Sp values, suggestive of bone resorption, which can be visually observed in Figure 6. Although not statistically significant, lower BV/TV mean percentage in vibrated pigs also confirms a bone resorption response to vibration. WBV did not significantly change any

cortical bone parameters, which is not surprising, considering that trabecular bone is more metabolically active than cortical bone in maintaining mineral homeostasis (Clarke, 2008). Trabecular bone also has a greater surface-to-volume ratio that is 10 times higher than cortical bone making it more sensitive to early biochemical changes in bone metabolism (Kim and Park, 2013). Therefore, a more pronounced effect of bone resorption would be expected for trabecular bone (Gonzalo et. al. (2018). These findings have been corroborated in sheep vibrated for 20 min/day with low-level (0.3g), high-frequency (30 Hz) mechanical vibration (Rubin et al., 2002). No change was observed in cortical bone by pQCT, but changes in trabecular bone remodeling were evident (C. Rubin et al., 2002). Furthermore, max RBAE values for the medial and lateral cortices were not significantly different between vibrated pigs and controls. However, this could be attributed to the anatomy of the pig's lower limb and tendency for the 2nd metacarpal bone to interfere with accurate readings of cortices in vivo for RBAE values for the 3rd metacarpal bone from a radiograph. In vivo studies using radiography to determine RBAE values should avoid metacarpal bones and consider other long bones such as the humerus or femur. Collectively, whole body vibration did not have a discernible osteogenic effect on bone in the pig when analyzed with imaging techniques.

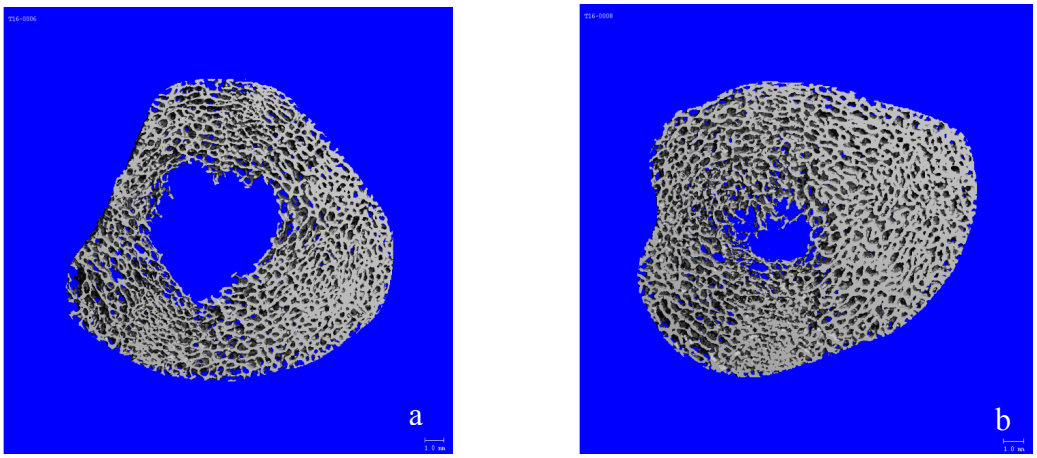

Figure 6. Micro-computed tomography (CT) image of (a) pig (vibrated, adequate diet) with decreased trabecular number (TbN.) and increased trabecular separation (Tb.Sp) compared to (b) pig (not vibrated, adequate diet).

Skeletal maturation in the growing animal is achieved through bone linear growth and bone mineral accrual. Bone remodeling functions to renew the skeleton with the coupled action of osteoclasts and osteoblasts (Allen and Burr, 2014), however, bone modeling occurs predominately during growth and functions to adapt bone to physiological influences or mechanical forces (Seeman, 2009). During bone modeling, bone adapts via independent actions of osteoblasts and osteoclasts in response to biomechanical forces (Clarke, 2008). Mechanical loading can inhibit bone resorption and stimulate formation locally in the modeling bone (Hillam and Skerry, 1995). These effects were observed in this study with growing pigs. Mean concentrations of CTX-I were lower in pigs receiving whole body vibration at 30 d and 60 d indicating bone resorption was reduced in those undergoing mechanical loading from the vibration. The adaptive response of bone to mechanical loading induces bone formation and inhibits resorption (Hillam and Skerry, 1995) and is a localized effect from strain (Sugiyama et al., 2010). Mean concentrations of bone formation marker osteocalcin were higher in vibrated pigs at 30 d and 60 d, and although not statically significant, might be indication of bone formation, also confirming

a local adaptive response. Certainly, more animal units in this study could have led to a more discernable outcome for WBV.

Growth performance characteristics of all four groups were similar. Eklou-Kalonji et al., (1999) reported similar findings when testing deficient Ca levels in the diet. Health, daily gain, feed conversion, and lean meat content has been satisfactory in many studies testing varying levels of Ca and P deficiency. Whereas, bone growth as well as mineral deposition is more influenced by variations in the supply of calcium and phosphorus. Growth and carcass characteristics may not be ideal parameters to test whereas testing bone parameters may be more beneficial (Nielsen, 1972).

Some of the more sensitive testing measures (microCT and bone biomarkers) of bone pointed towards bone resorption in the bone remodeling process due to whole body vibration. Given the duration of this 60 d study, the mechanical stimulus from vibration may not have been long enough to fully measure bone formation coupled with the initial bone resorption. Each remodeling cycle starts with bone resorption, which takes approximately 2 to 4 wk. Upon reversal, bone formation then takes approximately 4 to 6 mo to complete (Clarke, 2008). Also, perhaps the influence of decreased activity from housing in individual crates could have also contributed to increased bone resorption, as characterized by osteopenia during immobilization (Bloomfield, 2010). Further studies should focus on longer durations to capture the entire bone remodeling response and increase detailed knowledge about the bone remodeling timeline in the pig. When possible, studies should implement in vivo microCT analysis of bone to better quantify changes over time.

Additionally, the level of frequency and amplitude applied to the skeleton by WBV in this study may not have been sufficient to elicit a measurable bone remodeling response in cortical bone with the techniques utilized. Bone tissue will adapt if forces cause sufficient deformation, but if those forces are lacking, bone will not respond to increase its resistance to deformation (Frost et al., 1998). Every day activity has been shown to be characterized more as low amplitude high frequency on the bone (Fritton et al., 2000). Experiments testing WBV at a low amplitude, high frequency have been done in other species including sheep (Rubin et al., 2001a; Rubin et al., 2001b; C. Rubin et al., 2002; Clinton Rubin et al., 2002), rats (Flieger et al., 1998; Oxlund et al., 2003) and mice (Xie et al., 2006). In growing mice (8 wk old), vibration exposure of short durations of low magnitude and high frequency inhibited trabecular bone resorption indicating a maintenance of current bone status (Xie et al., 2006). Collectively, these studies demonstrate that the low magnitude high, frequency vibration used in this study should be effectively transmitted to the bone to stimulate an adaptive response.

Normal physiological responses of bone to a low Ca, P diet were observed in this study. And, although WBV did not elicit an osteogenic response, early indications of a local adaptive response were observed. The frequency and amplitude applied in this study was likely sufficient to elicit a bone remodeling response, the number of analyzed may also have been insufficient and certainly the duration of the study did not capture a full bone remodeling cycle.

CHAPTER IV

RESPONSE OF SERUM BIOMARKERS OF BONE METABOLISM AND BONE MINERALIZATION TO WHOLE BODY VIBRATION IN THIRD METACARPAL BONE OF STALLED YEARLING HORSES

INTRODUCTION

The strength and longevity of the performance horse has long been an area of interest for horse researchers, managers, and owners alike. Developing management practices to mitigate mechanical breakdown in the athletic horse is of great importance for the well-being of the animal, and also economics of the horse industry. Distal limb fractures are a common cause of career termination for many performance horses, including racehorses (Norwood, 1978; Verheyen and Wood, 2010). The longevity of the performance horse is largely dependent upon the quality of the appendicular skeleton. Numerous studies have focused on the epidemiology of fatal and non-fatal fractures in the racehorse (Mundy, 1997; Parkin, 2008). There have been several identifiable contributing factors and the two factors with the most relevance to this study are age and bone maturity and strength. The impact of age on soundness in the racehorse has been studied at length (Robinson et al., 1988; Mohammed et al., 1991; Wilson et al., 1996; Carrier et al., 1998; Williams et al., 2010). Two-year-old Thoroughbreds undergoing hard training experience higher incidents of unsoundness when their distal radial epiphysis has not yet closed (Mason and Bourke, 1973). Mixed perceptions exist about training and competing with horses as a long yearling and/or two-year-olds and its impact on bone development and injury. Evidence from the Poland racehorse industry has shown that horses first started as

two year olds achieved a longer race career than those started as three years olds (Sobczyńska, 2007). Additionally, Henley et al. (2006) showed that risk of fatal injury in racing increases with age.

Training and competition of the young horse is common, which can result in increased strain on developing skeletal structures. Increasing bone strength, especially in the young, growing horse has the potential to decrease failure of the skeletal system from mechanical stress due to training and competition. Horses experienced fewer injuries when they had greater cortical mass in the lateral and medial aspects of the third metacarpal, relative to the palmar aspect, at the commencement of race training in a large study conducted by Nielsen et al. (1997). Often these failures happen early in the training, as more demands are placed on a skeletal system that has not yet adapted to the new strain of training. Adaptation to intense exercise of the third metacarpal bone in the 2 year old horse has been largely attributed to changes in modeling rather than remodeling (McCarthy and Jeffcott, 1992; Firth et al., 2005). Upon some level of strain, bones undergo a remodeling process that is characterized by an increase in bone resorption. However, it has been demonstrated that porosity is lower at sites subjected to repetitive high magnitude loading (Whitton et al., 2010). During remodeling, resorption is followed by a period of bone formation, which ultimately results in a stronger bone to withstand strain. Resorption of bone is generally at its highest in the horse at about the same time the cardiovascular system begins to reach a level of fitness indicating adjustment to training demands (Nielsen et al., 1998). During this point of skeletal weakness, but cardiovascular fitness, training usually intensifies resulting in an increased chance of fatigue damage to the bone.

Over the past 30 years, whole body vibration (WBV) has received considerable attention in the research community as a means to improve bone mass and strength and there have been numerous studies to determine the effects of WBV on athletic performance and well-being (Hortobágyi et al., 2015). Whole body vibration is a therapy that exposes an individual to mechanical oscillations typically by standing on a vibrating platform. Some studies have shown WBV to have an osteogenic effect and increase bone quality and quantity in humans and rodents (Oxlund et al., 2003; Verschueren et al., 2004; Gilsanz et al., 2006; Xie et al., 2006; Pasqualini et al., 2013; Vanleene and Shefelbine, 2013). It is also widely used in the equine industry, especially with athletes, and has anecdotal evidence of positive effects on performance and health. However, limited research in the horse is available to support the idea of using WBV as a therapy or treatment in the care of the horse.

Whole body vibration is a non-invasive and relatively easy therapy to implement to economically manage an equine athlete. It could potentially be utilized to increase bone remodeling to ensure the skeletal system has adapted before extensive training and competition is superimposed on growth and development, limiting the probability of injury and career termination. This study is designed to address the question of whether WBV is an effective practice to increase bone density in young, growing horses.

Therefore, the objective of this study was to determine if WBV stimulation improves bone mass in young horses before early phase training through analysis of radiographic bone aluminum equivalent (RBAE) values and serum biomarkers, as indication of bone remodeling.

MATERIALS AND METHODS

Animals

Yearling, Quarter Horses (n=20; 11.4 to 15.5 mo of age; mean = 13.5 mo) with initial BW ranging from 271.8 kg to 347.7 kg (mean = 313.8 kg) and final BW ranging from 312.7 kg to 395.5 kg (mean = 355.8 kg) were used in this study. Horses belonged to Texas A&M University or were leased from private individuals. Horses were housed in 10×10 ft stalls at the Dick Freeman Equestrian Center. All 20 horses received 30 min of individual, free turnout time in a 30×30 ft dry lot 5 d/wk. Individual housing allowed for precise measurement of dietary intake. Horses were acclimated to diet and stalling regimens over a 2-wk period prior to the study. Use of animals was approved by the Texas A&M University Institutional Agricultural Animal Care and Use Committee using guidelines set forth by the Federation of Animal Science Societies (2016).

Diet

Diet consisted of forage and pelleted concentrate. Forage offered was Coastal Bermuda grass hay (92.7% DM). Concentrate fed was a 14% crude protein pelleted feed (Table 9) (Producer's Cooperative Association, Bryan, Texas). Samples were obtained by random core sampling of forage. Samples were submitted to a commercial laboratory for analysis (Table 10) (Dairy One, Ithaca, New York). Horses were fed a diet providing 100% NRC recommendation for DE and 110% NRC recommendation for Ca, P and protein. Horses had ad libitum access to water and a mineralized salt block throughout the experiment. Refusals and wastage were collected, dried and measured to subtract from intakes recorded.

Table 9. Concentrate nutrient analysis

Components[1]	As Fed
Crude Protein (%)	14.4
Lysine (%)	0.70
Fat (%)	6.0
ADF (%)	12.2
NDF (%)	23.9
Calcium (%)	0.83
Phosphorus (%)	0.53
Magnesium (%)	0.22
Potassium (%)	1.04
Sodium (%)	0.58
Iron (ppm)	146
Zinc (ppm)	115
Copper (ppm)	47
Manganese (ppm)	129
Horse DE, Mcal/lb	1.4

[1]Acid detergent fiber, ADF; neutral detergent fiber, NDF; digestible energy, DE.

Table 10. Coastal bermudagrass hay nutritional analysis

Components[1]	As Fed[2]	Dry Matter[2]	As Fed[3]	Dry Matter[3]
Moisture (%)	7.3	-	8	-
Dry Matter (%)	92.7	-	92	-
Crude Protein (%)	9.9	10.7	14	15.2
Adjusted Crude Protein (%)	9.9	10.7	14	15.2
ADF (%)	39.4	42.5	32.2	34.8
aNDF (%)	69.5	75	62.5	67.9
NFC (%)	4.7	5.1	7.1	7.7
TDN (%)	51	55	52	57
NEL, Mcal/lb	0.34	0.36	0.42	0.46
NEM, Mcal/lb	0.42	0.46	0.45	0.49
NEG, Mcal/lb	0.19	0.21	0.22	0.24
Relative Feed Value	-	69	-	85
Calcium (%)	0.35	0.37	0.35	0.38
Phosphorus (%)	0.2	0.21	0.2	0.21
Magnesium (%)	0.16	0.18	0.18	0.2
Potassium (%)	0.65	0.7	0.69	0.75
Sodium (%)	0.37	0.39	0.46	0.5
Iron (ppm)	228	246	207	225
Zinc (ppm)	20	21	26	28
Copper (ppm)	6	7	11	12
Manganese (ppm)	238	257	170	184
Molybdenum (ppm)	0.6	0.6	0.4	0.5
Horse DE, Mcal/lb	0.74	0.8	0.81	0.88

[1]Acid detergent fiber, ADF; neutral detergent fiber, aNDF; non-fibrous carbohydrate, NFC; total dietary nitrogen, TDN; net energy for lactation, NEL; net energy for maintenance, NEM; net energy for gain, NEG; digestible energy, DE.

[2]Coastal Bermudagrass hay sample submitted for analysis May 4, 2016.

[3]Coastal Bermudagrass hay sample submitted for analysis September 16, 2016.

Treatments

Horses were blocked by weight and balanced for gender (geldings = 4; fillies = 16), then assigned to either receive WBV or serve as a control (not receiving WBV). Horses receiving vibration treatment (n = 10) stood on a vertical whole body vibration plate (Equivibe, Lincoln, NE), which is a stationary platform 4 inches off the ground, for 30

min/d, 5 d/wk for 120 d. Horses undergoing vibration treatment were walked to the vibration plate Monday-Friday. Vibration was a low magnitude (1-2mm) and high frequency (50 Hertz). A freestanding stock surrounded the vibration plate to ensure the horse remained standing and correctly positioned. The vibration plate was rotated 180 degrees every three weeks to ensure both sides of the plate was equally represented in the vibration treatment.

Physical Measurements

Horses were weighed with a digital, platform scale on Day 0, 30, 60, 90, and 120.

Radiographic Bone Aluminum Equivalency (RBAE) Measurements

Digital radiographs of the left, third metacarpal bone and aluminum step wedge were taken on Day 0, 30, 60, 90, and 120 using a Portable Veterinary X-ray unit (MinXray, Inc, Northbrook, IL). A dorsal-palmar view was taken at a focal distance of 26 cm and exposure of 76 kVp and 0.06 ms. Images were produced by standing the horse in a stock and placing a 10×10 inch cassette directly in line with the area of interest. An aluminum (AL) step wedge penetrometer of 11 steps ranging from 5 mm to 35 mm in 3mm increments was attached to each radiographic cassette on the same side. Aluminum step wedge was used to standardize readings and determine RBAE values using software validated by O'Connor-Robison and Nielsen (2013).

Original digital radiograph files were converted to 16-bit TIFF picture files using the open source software ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA.). Quantity One 1-D analysis software (BioRad, Hercules, CA) was used to analyze TIFF files. Images were rotated to orient the third metacarpal bone in a vertical position using the penetrometer as a guide. The Volume Rect

Tool was used to draw two boxes around the area of interest, the cortices 1 cm distal to the nutrient foramen of the diaphysis (Fig. 7). The same sized box was copied and pasted on each step of the AL step wedge (11 steps total) and on the cortical bone. Exact size of box was confirmed by identical Area given in the Volume Analysis Report. The Volume Analysis Report was used to obtain data from these boxes on the following parameters:

Volume (Intensity (INT)*mm^2)-Sum of the intensities of the pixels inside the volume boundary x area of a single pixel.

Area (mm^2)-The total area of the volume box you have drawn in mm^2.

Mean Value (INT)-The mean intensity of the pixels inside the volume boundary.

Min. Value (INT)-The value of the lowest intensity pixel in the volume.

Max. Value (INT)-The value of the highest intensity pixel in the volume

Density (INT/mm^2)-The total intensity of all the pixels in the volume divided by the area of the volume.

Values for each parameter were obtained from the boxes for each step of the AL step wedge and used to develop a best-fit linear equation to predict the values of the medial and lateral boxes of the cortical bone.

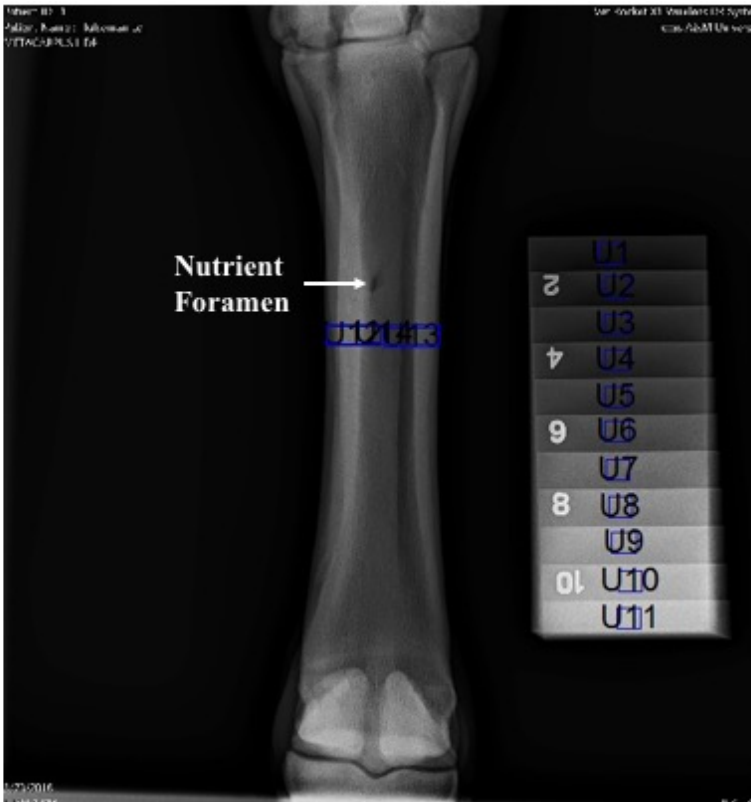

Figure 7. Digital radiograph of horse third metacarpal diaphysis next to aluminum step wedge. Cortices one cm distal to nutrient foramen were analyzed using Quantity One software to determine radiographic bone aluminum equivalency.

Serum Bone Biomarkers

Blood samples were collected on 0, 30, 60, 90, and 120 d via jugular venipuncture using a 3.81-cm, 20-gauge needle. A 10mL purple-top vacutainer (EDTA) and a 10mL red-top vacutainer (no additive) were collected. Red-top tubes were allowed to clot at room temperature for 20 min, then transferred to an ice chest before being centrifuged at room temperature (<6 hr elapsed from time of collection to harvest). Purple-top tubes were immediately transferred to an ice chest before being centrifuged at 2000 x g at 4°C for 20 min. Serum and plasma were collected and stored at -80°C until analysis. Serum was analyzed for biomarkers to measure the rate of bone turnover (remodeling). Serum concentration of osteocalcin was determined via rat osteocalcin enzyme immunoassay

(EIA) kit (Immunodiagnostic Systems Holdings PLC, Gaithersburg, Maryland) as indicator of osteoblast activity indicating bone osteogenesis (formation). Serum concentration of carboxy-terminal collagen crosslinks (CTX-1) was determined via human C-telopeptide of collagen (CTX) ELISA kit (Immunodiagnostic Systems Holdings PLC, Gaithersburg, Maryland) as an indicator of bone resorption. Kits were validated by dilutional parallelism and intra and inter assay variability. Evaluated samples were assayed within the linear range.

Statistical Analysis

Data was analyzed as a completely randomized design using the MIXED procedure of SAS (SAS Institute INC., Cary, NC) with horse as the experimental unit. Day of RBAE measurement served as the repeated measure. The RBAE values were determined by developing a linear regression equation from the aluminum step-wedge penetrometer of varying steps of optical density. The aluminum step-wedge is a standard for each radiograph and therefore an equation was developed for each radiograph. The equation was then used to solve for the bone optical density of each cortices. Statistical significance was determined at $P < 0.05$ and $P < 0.10$ was considered a trend.

RESULTS

Radiographic Bone Aluminum Equivalency (RBAE)

There were no vibration × day interactions ($P > 0.05$) on max RBAE values for the medial and lateral cortices of the third metacarpal bone in yearling horses (Table 10). A significant difference was not observed between max RBAE values for the medial cortices in horses that were vibrated compared to controls. However, horses that were vibrated tended to have increased ($P = 0.062$) max RBAE values for the lateral cortices compared to controls (Figure 8). Mean RBAE max values for medial and lateral cortices were unchanged from d 0 to 120 ($P > 0.223$).

Table 11. Main effects of whole body vibration (WBV) on radiographic bone aluminum equivalence (RBAE) max values of medial and lateral cortices of third metacarpal bone in yearling horses[1]

	WBV			*P*-value[2]				
	Yes	No	SEM	WBV × Day, Linear	WBV × Day, Quadratic	WBV	Day, Linear	Day, Quadratic
Medial Cortices[3,4]								
d 0	25.7	25.4	0.792	0.654	0.227	0.436	0.223	0.341
d 30	24.3	25.6						
d 60	23.8	25.7						
d 90	26.7	25.9						
d 120	23.9	23.8						
Lateral Cortices[3,4]								
d 0	22.4	22.1	0.928	0.480	0.730	0.062	0.973	0.906
d 30	21.4	20.2						
d 60	21.3	21.0						
d 90	24.4	21.8						
d 120	21.6	20.5						

[1]A total of 20 yearling horses (average initial BW = 314kg) were used in a 120-d trial. Horses were individually housed, n = 10 (vibrated) n = 10 (control). Means differ at a time with no letters in common $P < 0.05$.

[2]No WBV x day interaction was observed ($P > 0.05$).

[3]Day 0 radiographic bone aluminum equivalency (RBAE) max values used as a covariate.

[4]Radiographic bone aluminum equivalency (RBAE) max values in mm aluminum.

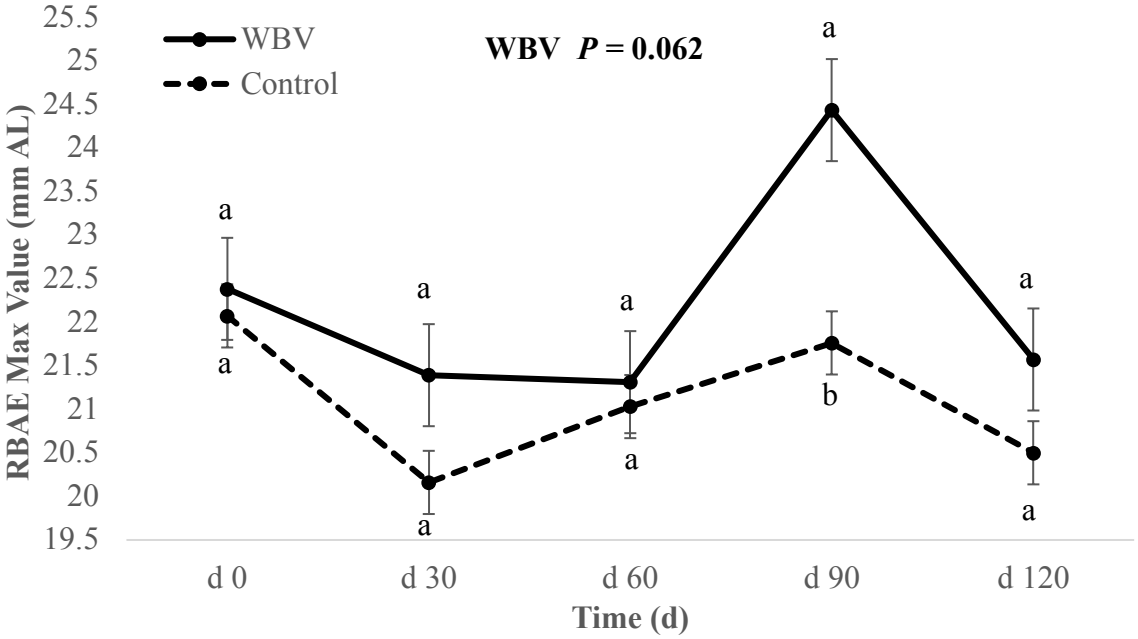

Figure 8. Changes in RBAE max values for the lateral cortices over time (d) for horses receiving vibration (Treatment) and those not receiving vibration (Control). Means differ at a time with no letters in common; lower case, $P < 0.05$.

Serum Bone Biomarkers – Osteocalcin (OC) and Carboxy-Terminal Collagen Crosslinks (CTX-I)

There were no vibration x day interactions ($P > 0.14$) on bone turnover biomarkers osteocalcin and carboxy-terminal collagen crosslinks (CTX-1; Table 11). Horses receiving vibration treatment had decreased ($P = 0.003$) CTX-1 concentrations compared to horses that were not vibrated (Figure 9). There was no difference in osteocalcin concentrations between horses that were vibrated and controls except at 90 d (Figure 10). Mean concentrations for CTX-1 and osteocalcin increased (quadratic, $P = 0.0002$ and linear, $P = 0.001$, respectively) in horses from d 0 to 120.

Table 12. Main effects of whole body vibration (WBV) on bone turnover biomarkers in yearling horses[1]

	WBV			*P*-value[2]				
	Yes	No	SEM	WBV × Day, Linear	WBV × Day, Quadratic	WBV	Day, Linear	Day, Quadratic
CTX-1[3,4]								
d 0	0.26	0.26	0.381	0.143	0.622	0.003	<0.0001	0.0002
d 30	0.31	0.37						
d 60	0.35	0.40						
d 90	0.41	0.51						
d 120	0.31	0.40						
Osteocalcin[3,5]								
d 0	672.9	655.9	76.6	0.434	0.873	0.752	0.001	0.658
d 30	536.8	654.5						
d 60	752.7	765.2						
d 90	897.5	696.6						
d 120	823.3	841.0						

[1]A total of 20 yearling horses (average initial BW = 314kg) were used in a 120-d trial. Horses were individually housed, n = 10 (vibrated) n = 10 (control). Means differ at a time with no letters in common $P < 0.05$.

[2]No WBV x day interaction was observed ($P > 0.05$).

[3]Day 0 biomarker values used as a covariate.

[4]Bone resorption biomarker (ng/mL).

[5]Bone formation biomarker (ng/mL).

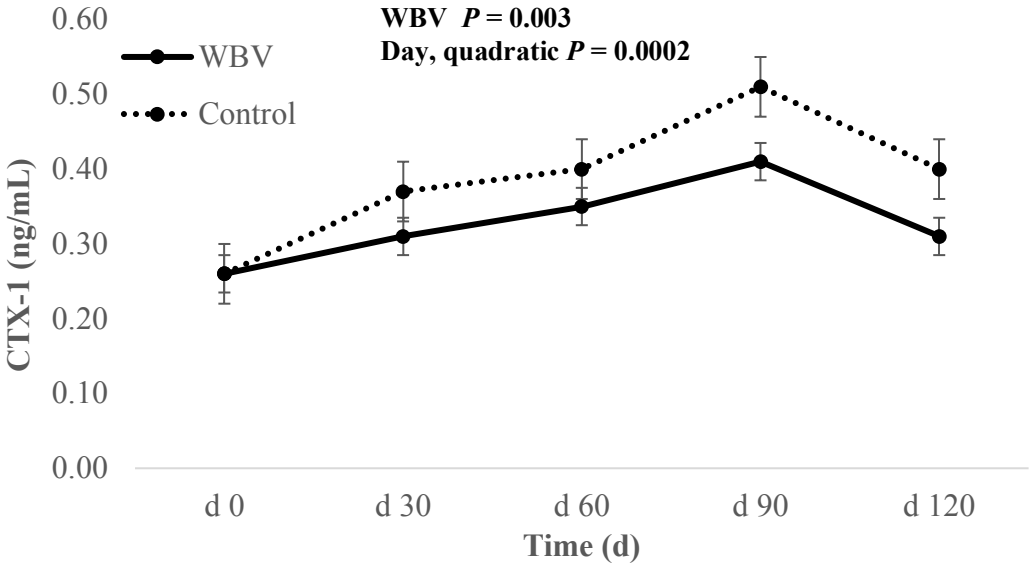

Figure 9. Changes in serum concentrations of CTX-1 (ng/mL) over time (d) for horses receiving WBV (treatment) and those not receiving vibration (control). Means differ at a time with no letters in common $P < 0.05$.

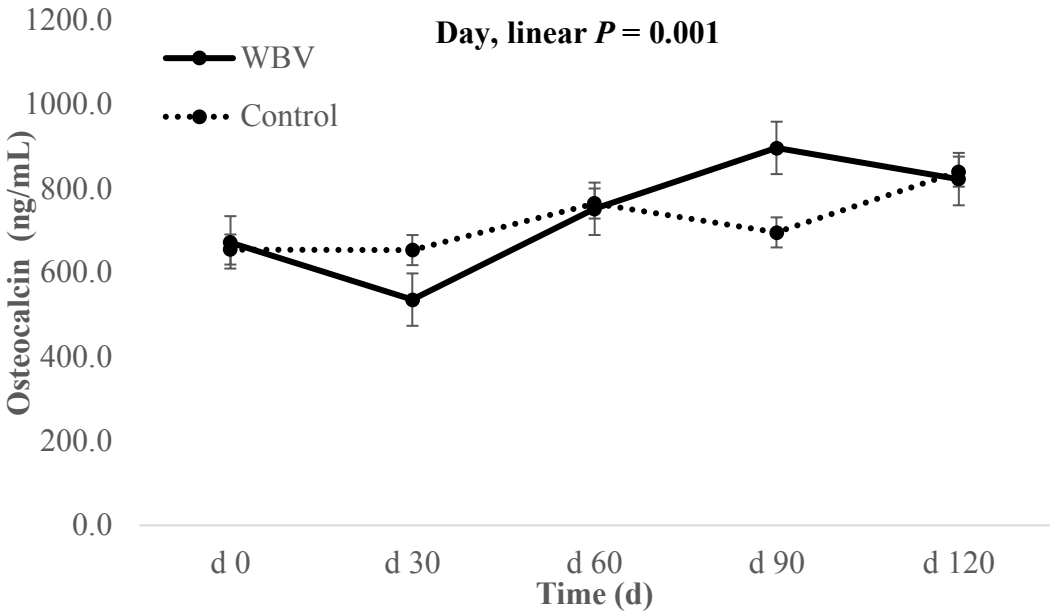

Figure 10. Changes in serum concentrations of osteocalcin (ng/mL) over time (d) for horses receiving WBV (treatment) and those not receiving vibration (control). Means differ at a time with no letters in common $P < 0.05$.

DISCUSSION

The primary objective of this study was to determine the effects of whole body vibration (WBV) on radiographic bone mass and composition in yearling horses. We hypothesized that WBV would have an anabolic effect on bone and therefore improve bone quality and quantity. Bone quality is the totality of properties that make bone resist to fracture, such as its microarchitecture, accumulation of microscopic damage, quality of collagen, mineral crystal size, and bone turnover (Bouxsein, 2003; Fyhrie, 2005). Robust bones defy failure while fragile bones are more prone to fail (Fritton J and Schaffler M, 2008).

Bone turnover changes in response to WBV are mixed across human and rodent studies. Some literature has indicated bone-strengthening effects from WBV (Flieger et al., 1998; Rubin et al., 2001; Oxlund et al., 2003; Verschueren et al., 2004; Gilsanz et al., 2006; Gusi et al., 2006; Xie et al., 2006) while others have shown no effect (Torvinen et al., 2003). In this study, WBV treatment tended to elicit a response change in the bone mineral content of the lateral cortices. Max RBAE values were greater in lateral cortices of horses vibrated than controls. Medial cortices, however, were not significantly different between vibrated horses and controls. Numerical values for means of max RBAE values in this study would indicate that the greatest strain from whole body vibration was over the medial aspect of the cortex, with higher means than the lateral aspect throughout the entire study. This is contradictory to strain levels recorded by Rubin et al. (2013), who reported the posterior/lateral cortex was consistently exposed to the greatest magnitude normal and shear strain, while the anterior/medial cortex was consistently exposed to the lowest strain, indicating a higher bone mineral content would be expected in the lateral cortices

compared to the medial cortices. However, their study was severely limited by only assessing three animals (Rubin et al., 2013). Others have confirmed increased bone quality of the medial cortices in comparison to other areas, such as the lateral and palmar aspects of the third metacarpal bone (Nielsen et al., 1997). The largest recorded strains during galloping occur in the dorsal and medial cortices of the third metacarpal bone (Gross et al., 1992), therefore it would be expected that exercise would cause greater mineralization to occur in those areas. Weanlings exercised at a medium trot for up to 20 min, 5 d/wk tended to have an increased radiographic bone density of the medial cortices compared to those that did not receive exercise (Raub et al., 1989). Additionally, the third metacarpal bone appears to be designed to resist axial compression and mediolateral bending, as it is has a greater stiffness than the anterior/posterior plane (Piotrowski et al., 1983).

The data reported in this chapter supports the conclusions reached by Hiney et al. (2004). Similar to ours, that study observed that changes in RBAE values may have been due to formation of new bone rather than increased mineralization of preexisting bone, which is often the case with bone modeling in young, growing animals. Skeletal maturation in the growing animal is achieved through bone linear growth and bone mineral accrual. Bone remodeling functions to renew the skeleton with the coupled action of osteoclasts and osteoblasts (Allen and Burr, 2014), however, bone modeling occurs predominately during growth and functions to adapt bone to physiological influences or mechanical forces (Seeman, 2009). During bone modeling, bone adapts via independent actions of osteoblasts and osteoclasts in response to biomechanical forces (Clarke, 2008). Mechanical loading, such as exercise, can inhibit bone resorption and stimulate formation locally in the modeling bone (Hillam and Skerry, 1995). These effects were observed in

this study with growing horses. Mean concentrations of CTX-I were lower in horses receiving whole body vibration from 30 d to 120 d of the study, indicating bone resorption was reduced in those undergoing mechanical loading from the vibration. The adaptive response of bone to mechanical loading induces bone formation and inhibits resorption (Hillam and Skerry, 1995) and is a localized effect from strain (Sugiyama et al., 2010). Bone formation is confirmed in this study by the numerical increase in osteocalcin concentrations from baseline in the vibrated horses, although not statistically significant.

Many horse studies focus on the cortical bone, however, it has been shown that trabecular bone is more metabolically active than cortical bone in maintaining mineral homeostasis (Clarke, 2008). Trabecular bone also has a greater surface-to-volume ratio, being 10 times higher than cortical bone. As a result, trabecular bone is far more sensitive to early biochemical changes in bone metabolism (Kim and Park, 2013). Therefore, a more pronounced effect of bone resorption would be expected for trabecular bone (Gonzalo et. al. 2018). These findings are corroborated in sheep vibrated for 20 min/day with low-level (0.3g), high-frequency (30 Hz) mechanical vibration (Rubin et al., 2002). No change was observed in cortical bone by peripheral quantitative computed tomography (pQCT), but changes in trabecular bone remodeling were evident (Rubin et al., 2002). Trabecular bone has been indicated as more suitable to study the effects of external factors as turnover time was found to be 8 times as fast as in cortical bone (Scotti and Jeffcott, 1988). Further studies of changes in equine bone would be well served to incorporate more sensitive and detailed imaging techniques that allow for the assessment of trabecular bone, in addition to cortical bone. Because radiography and dual energy X-ray absorptiometry scanning are planar techniques, they cannot be used to illustrate and quantify features of bone

development except serial measurement of linear dimensions (Davies et al., 1999) and areal density (Fujita, 2002). Bone mineral density only characterizes approximately 70 to 75% of bone strength (Ammann and Rizzoli, 2003) and therefore other parameters of bone such as macro- and micro-architecture and tissue quality are important to measure, which is possible with more advanced technologies (Burghardt et al., 2011). Use of more sensitive modalities to detect changes may be the difference between diagnosing microdamage and mitigating catastrophic failure (Morgan et al., 2006). Even so, digital radiography still has distinct advantages over other methodologies by being inexpensive, portable, and easy to use in the field setting (Bowen et al., 2013). Additionally, RBAE values have been highly correlated with bone mass (Meakim et al., 1981) which, in turn, has been described as the best measurable determinant of bone strength in horses.

To the author's knowledge, no equine study has used the rat EIA for osteocalcin measurements in the horse. In humans, a strong correlation was obtained between CTX-I concentrations obtained with serum Serum CrossLaps ELISA and sandwich CTX-I ECLIA (Christgau et al., 1998). Carstanjen et al. (2004) then found good correlation between expected equine serum CTX-I concentrations and measured serum CTX-I concentrations by CTX-I ECLIA. This study used the Serum CrossLaps ELISA to measure CTX-I concentrations, and therefore, concentrations obtained in this study are close in range to those obtained by Carstanjen et al. (2004) for similar age of horses. Many bone biomarker results in the horse are only reported as percentage change from baseline, thereby making it difficult to make inferences about actual concentrations measured between this study and others. The observed increases in CTX-1 concentrations across all horses from 0 d to 90 d in this study could be indicative of normal physiological response to disuse. The influence

of decreased activity from housing in individual stalls could have contributed to increased bone resorption, as characterized by osteopenia during immobilization (Bloomfield, 2010). Confinement of foals inhibited musculoskeletal development, but brief exercise in addition to confinement or continual pasture exercise resulted in bone properties that were more resistant to deformation (Barneveld and Weeren, 2010). Many investigators have demonstrated that confining a horse under the age of 2 yr to a stall without exercise results in increased bone resorption and therefore decreased bone mineral content, decreased bone formation, and delayed musculoskeletal development (Mäenpää et al., 1988; Hoekstra et al., 1999; Bell et al., 2001; Barneveld and Weeren, 2010).

While osteocalcin levels in this study did not confirm a decrease in bone formation, other studies have found no difference to be noted in osteocalcin concentrations in yearlings stalled for 84 d in comparison to those turned out on pasture (Hoekstra et al., 1999). Additionally, urinary concentrations of deoxypyridinoline, a bone resorption marker, were greater at d 28 in horses housed in stalls than on pasture. In this same study, RBAE values for medial and lateral cortices also tended to be lower in horses confined to a stall than those turned out to pasture (Hoekstra et al., 1999). Additionally, a study in 2017 compared horses exercised to those exercised and vibrated. Results indicated no influence of WBV on RBAE values of any bone cortices or bone turnover biomarkers, pyridinoline cross-links and osteocalcin. However, there was a period effect of a decrease in RBAE lateral cortices, which the author also contributed to a likely effect of stalling (Maher et al., 2017). Medial and lateral cortices max RBAE means were lower from 0 d to 120 d in all horses in this study confirming that housing yearling horses in stalls, with limited access to exercise, may decrease bone mineral content.

Finally, the level of frequency and amplitude applied to the skeleton by WBV in this study may not have been sufficient to elicit a measurable bone remodeling response in cortical bone with the techniques utilized. Bone tissue will adapt if forces cause sufficient deformation, but if those forces are lacking, bone will not respond to increase its resistance to deformation (Frost et al., 1998). Hulak et al. (2015) compared WBV to light exercise in adult horses (mean age 17±4 yr) that were stalled. Vibrated horses stood on a vibration plate for 45 min at 50 Hz, 5 d/wk, which was a slight increase in duration from this study at 30 min per vibration session. Hulak et al. (2015) exercised a second group of horses on a mechanical panel exerciser for 60 min, 6 times per wk. After a 28-d treatment period, RBAE determined BMC to increase in both groups concluding that WBV maintained BMC in the same way light exercise would in stalled horses (Hulak et al., 2015). Every day activity has been shown to be characterized more as low amplitude high frequency on the bone (Fritton et al., 2000). Experiments testing WBV at a low amplitude, high frequency have been done in other species including sheep (Rubin et al., 2001a; Rubin et al., 2001b; C. Rubin et al., 2002; Clinton Rubin et al., 2002), rats (Flieger et al., 1998; Oxlund et al., 2003) and mice (Xie et al., 2006). In growing mice (8 weeks old), vibration exposure of short durations of low magnitude and high frequency inhibited trabecular bone resorption indicating a maintenance of current bone status (Xie et al., 2006). Collectively, these studies demonstrate that the low magnitude, high frequency vibration used in this study should be effectively transmitted to the bone to stimulate an adaptive response.

Responses observed from 0 d to 60 d in RBAE values for both medial and lateral cortices closely align with a study conducted by Nielsen et al. (1997) in 53 Quarter Horses put into race-training at 18 mo of age. A decrease in both medial and lateral cortices RBAE

values were noted from 0 d to 62 d, with 0 d being the commencement of training. In this study, RBAE values also decreased from 0 d to 60 d. The decline in RBAE values are indicative of a bone remodeling response to exercise. Each remodeling cycle starts with bone resorption, which takes approximately 2 to 4 wk (Clarke, 2008). Bone resorption then transitions to formation through a reversal phase that can last up to 4 or 5 wk (Hadjidakis and Androulakis, 2006), and finally bone formation takes approximately 4 to 6 mo to complete the remodeling cycle (Clarke, 2008). Given the duration of this 120 d study, the mechanical stimulus from vibration may not have been long enough to fully measure bone formation coupled with the initial bone resorption. Further studies should focus on longer durations to capture the entire bone remodeling response and increase detailed knowledge about the bone remodeling timeline in the young horse.

CHAPTER V

SUMMARY

Mechanical loading of the skeleton can have positive effects on bone by increasing mass and strength. Whole body vibration (WBV) is a relatively easy therapy that has the potential to load the skeleton to produce adaptive responses. It has been implemented in the equine industry to manage horse athletes, but also in human health as a method to counteract bone loss, such as with osteoporosis. This research project assessed the purportedly positive effects of whole body vibration (WBV) on bone density in the horse. Despite the prevalence of claims that WBV therapy improves physiological functions or increases bone density in horses, very little research exists to substantiate these claims.

A preliminary trial was conducted with growing pigs, which began to define the effects of WBV and Ca and P levels on bone mass and composition. Since horses are not routinely slaughtered in the United States, the preliminary study in pigs allowed for utilization of additional bone testing techniques, including micro computed tomography (CT), which was performed on the excised third metacarpal bone.

This study defined the outcomes of bone remodeling in response to WBV as a form of mechanical loading. In both studies, bone biomarkers osteocalcin (OC) and carboxy-terminal collagen crosslinks (CTX-I) were utilized to measure serum changes in bone turnover. Future studies on bone, especially in equine studies, should consider use of bone biomarkers to measure changes.

MicroCT was utilized in the pig study to provide detailed information regarding bone microstructure and changes in both trabecular and cortical bone in response to WBV.

Equine studies would be well served to evaluate changes in trabecular bone, in addition to cortical bone, to establish an overall perspective of bone turnover. Unfortunately use of computed tomography (CT) is limited in the horse, and use of microCT necessitates an invasive bone biopsy. However, advancement in CT technology for use in horses is on the horizon. In the meantime, radiographic bone aluminum equivalency (RBAE) values demonstrate measurable changes in cortical bone of the horse, but not necessarily in the pig when evaluating the third metacarpal bone.

Studies utilizing young, growing animals to evaluate changes in the skeleton need to address changes observed due to modeling and remodeling. Both are active parts of the developing skeleton and must be considered when drawing conclusions based on data collected. Reponses measured in this study pointed toward changes observed in both modeling from growth and remodeling from mechanical stimulation.

Implications

There are still clear limitations to fully defining the effects of WBV in the horse, especially its influence on the skeleton. It continues to be widely used in the industry as anecdotal evidence grows in support of its positive effects. Whole body vibration has potential to be useful in equine therapy, however placebo controlled trials are lacking to fully define the effects of WBV.

Studies in humans have typically exposed patients to 6 mo to 1 yr of WBV, therefore the duration of this study may not have been long enough to fully capture the effects of WBV. However, the amount of time to skeletal maturity in animals, like the pig and horse, is substantially shorter than that in humans. A duration of 120 d in horses and

60 d in pigs was predicted to be adequate to measure a skeletal response to WBV. Future studies should consider a longer experimental duration to capture the full bone response.

The amplitude and frequency used in this study has shown to maintain bone mineral content in stalled horses (Hulak et al., 2015), however an osteogenic effect has yet to be demonstrated in horses. The level of strain induced by WBV at different amplitudes and frequencies on specific bones is an important factor that future studies should consider defining. The horse appendicular skeleton undergoes tremendous amounts of strain during fast locomotion and the skeletal response is osteogenic (Nunamaker et al., 1990; Hiney et al., 2004). Perhaps low amplitude, high frequency WBV may not be enough strain to elicit an osteogenic response in the horse. There are numerous combinations of strain, session length of WBV, and duration of exposure to WBV that have yet to be studied. It would also be useful for future studies to measure the vibration intensity at certain locations on the vibration platform. Vibration across the platform may lack uniformity and therefore horse stance on the platform may influence how much strain is actually being transduced across the body.

Mechanical loading of the modeling (growing) skeleton may result in the best opportunity for improvement of skeletal strength. Modeling changes the shape of bones to better withstand mechanical forces placed on the skeleton (Seeman, 2009). Horses under the age of 2 yr experience a time of rapid growth and bone mineralization (El Shorafa et al., 1979), which is a window of opportunity for skeletal adaptation to loading. Future studies in growing horses need to more closely define outcomes that are a modeling response or a remodeling response.

Changes in trabecular bone, in addition to cortical bone, can be insightful to interpretation of bone response to loading. Future horse studies would be well served to define changes in trabecular bone. Unfortunately, methods for analyzing trabecular bone in the horse are limited. Use of computed tomography (CT) is expensive, complicated, and not practical. However, improvements of CT technology for use in horses are on the horizon, which may make it more accessible. In the meantime, careful considerations need to be implemented to minimize analytical variation in studies utilizing radiographs. Radiographs need to be consistently placed and executed from animal to animal. This includes capturing images at the same distance from each specimen and placing radiographic cassette in an immobile stand that allows for the consistent angles during radiography.

In vivo studies using radiography to determine RBAE values in pigs should avoid the metacarpal bones and consider using other long bones, such as the humerus or femur. The anatomy of the pig's lower limb results in a tendency for the 2nd metacarpal bone to interfere with accurate readings of cortices for the 3rd metacarpal bone from a radiograph. The 3rd metacarpal bone in the horse is an excellent specimen to evaluate bone changes with radiography due to minimal soft tissue and bone interferences, however it should be avoided in the pig.

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APPENDIX

Appendix Table 1. Radiographic bone aluminum equivalency (RBAE) max calculated values using aluminum step wedge with 1 to 11 steps. Values obtained from dorsal/palmar (DP) radiograph evaluating medial and lateral cortices of third metacarpal bone for each yearling horse at 0 d.

			Max. Value (INT)-The value of the highest intensity pixel in the volume Aluminum Step Wedge Steps 1 through 11 (mm)											Calculated Values						
Horse	Day	Vibration (A=Yes; B=No)	5	8	11	14	17	20	23	26	29	32	35	Difference first, last AL step	m	b	X-Medial Cortices	Y-Medial Cortices	X-Lateral Cortices	Y-Lateral Cortices
1	0	B	446	618	728	916	1130	1634	2201	2654	2971	3207	3382	2936	0.0088	4.0923	2292	24.2595	1577	17.9682
2	0	B	532	726	817	1069	1275	1899	2422	2857	3048	3272	3463	2931	0.0088	2.8332	2405	24.0749	2257	22.7677
3	0	A	392	504	692	880	991	1397	1984	2471	2930	3231	3586	3194	0.0084	5.4610	2419	25.7605	2012	22.3451
4	0	B	554	664	765	916	1126	1588	2161	2594	3083	3231	3398	2844	0.0088	3.9099	2627	27.0651	2078	22.2260
5	0	A	387	533	716	925	1211	1549	2147	2720	2964	3271	3419	3032	0.0086	4.5095	2615	26.9661	2208	23.4710
6	0	B	394	596	751	902	1242	1567	2156	2763	2968	3208	3397	3003	0.0087	4.1959	1858	20.3915	1744	19.3978
7	0	B	452	595	767	935	1164	1570	2161	2598	2927	3229	3383	2931	0.0089	3.9877	1948	21.3332	2352	24.9305
8	0	B	353	510	678	908	1124	1492	2149	2802	2893	3183	3400	3047	0.0085	4.8854	2401	25.3652	2057	22.4310
9	0	A	374	558	696	922	1117	1480	2124	2724	2955	3282	3395	3021	0.0085	4.7979	2819	28.8159	2164	23.2353
10	0	A	398	574	668	928	1143	1503	2140	2780	2952	3169	3438	3040	0.0086	4.6466	2212	23.6167	2081	22.4933
11	0	A	480	592	773	925	1277	1652	2214	2684	3122	3324	3430	2950	0.0086	3.9772	2188	22.8136	1992	21.1262
12	0	A	388	542	710	970	1141	1564	2146	2729	2967	3275	3390	3002	0.0086	4.5056	2745	28.1084	1836	20.2923
13	0	B	367	540	686	908	1125	1514	2117	2687	2976	3242	3476	3109	0.0085	4.8702	2119	22.8283	1703	19.3028
14	0	B	582	725	860	1080	1480	2250	2532	2881	3257	3356	3481	2899	0.0087	2.2918	3243	30.3875	2299	22.2092
15	0	B	448	560	730	897	1147	1556	2163	2606	2994	3247	3383	2935	0.0087	4.3704	2227	23.7753	1618	18.4688
16	0	B	512	656	790	999	1311	1752	2434	2850	3116	3320	3480	2968	0.0086	3.4674	3281	31.5861	3106	30.0863
17	0	A	544	734	827	1007	1384	1942	2472	2903	3143	3321	3466	2922	0.0087	2.8242	2742	26.6505	2097	21.0459
18	0	A	428	539	684	907	1105	1506	2099	2545	2963	3248	3418	2990	0.0087	4.6991	2330	24.8700	1728	19.6584
19	0	A	417	547	686	897	1145	1578	2158	2614	2975	3206	3385	2968	0.0087	4.4775	2455	25.8558	2442	25.7426
20	0	A	479	612	811	988	1341	1798	2428	2764	3030	3265	3422	2943	0.0088	3.2702	2708	27.0713	2508	25.3135

Appendix Table 2. Radiographic bone aluminum equivalency (RBAE) max calculated values using aluminum step wedge with 1 to 11 steps. Values obtained from dorsal/palmar (DP) radiograph evaluating medial and lateral cortices of third metacarpal bone for each yearling horse at 30 d.

			Max. Value (INT)-The value of the highest intensity pixel in the volume Aluminum Step Wedge Steps 1 through 11 (mm)											Calculated Values						
Horse	Day	Vibration (A=Yes; B=No)	5	8	11	14	17	20	23	26	29	32	35	Difference first, last AL step	m	b	X-Medial Cortices	Y-Medial Cortices	X-Lateral Cortices	Y-Lateral Cortices
1	30	B	358	552	670	868	1077	1499	2152	2584	2945	3207	3413	3055	0.0086	4.9147	2335	24.9647	1494	17.7432
2	30	B	151	473	673	882	1202	1623	2235	2841	3153	3395	3622	3471	0.0078	5.6360	2067	21.7641	1739	19.2048
3	30	A	384	594	731	946	1106	1634	2261	2668	2957	3307	3387	3003	0.0086	4.3530	2360	19.3373	2710	27.7041
4	30	B	324	559	733	944	1129	1541	2226	2746	3113	3318	3699	3375	0.0081	5.0985	2687	21.7626	1822	19.7875
5	30	A	242	472	642	874	1073	1403	2061	2605	2938	3279	3640	3398	0.0081	5.8209	2286	27.8022	1859	20.8996
6	30	B	145	354	572	813	1024	1325	1918	2582	3001	3300	3614	3469	0.0078	6.7066	2364	27.7766	1568	19.0020
7	30	B	321	534	685	891	1185	1635	2277	2665	3054	3413	3340	3019	0.0083	4.8202	2257	23.9058	1628	18.4122
8	30	B	357	522	699	892	1098	1532	2178	2625	2910	3214	3389	3032	0.0086	4.7484	2521	25.1750	2368	25.2096
9	30	A	302	526	704	877	1123	1569	2216	2623	2932	3310	3405	3103	0.0085	4.9009	3042	24.0394	2130	22.9625
10	30	A	292	512	650	810	988	1275	1926	2492	2975	3251	3661	3369	0.0081	6.1864	1822	26.5276	1801	20.7181
11	30	A	246	441	633	862	973	1459	2210	2649	2919	3186	3280	3034	0.0084	5.5659	2143	31.1780	1549	18.6077
12	30	A	287	542	715	892	1196	1602	2324	2832	3038	3311	3567	3280	0.0081	4.9647	2919	19.8045	2683	26.8172
13	30	B	264	501	670	850	1159	1521	2198	2650	2913	3275	3353	3089	0.0085	5.0716	2228	23.2543	1541	18.1465
14	30	B	377	592	764	965	1245	1706	2340	2775	3157	3320	3371	2994	0.0085	4.1214	2907	28.8568	1901	20.2303
15	30	B	355	521	697	887	1131	1547	2201	2621	2991	3210	3259	2904	0.0087	4.6269	2318	24.0277	2069	22.6432
16	30	B	322	561	710	928	1171	1619	2314	2745	3131	3273	3534	3212	0.0082	4.7855	2721	28.7423	1817	19.7595
17	30	A	296	512	658	891	1095	1635	2277	2648	3054	3413	3409	3113	0.0082	5.1714	2325	24.1829	1593	18.2366
18	30	A	332	539	689	902	1171	1631	2159	2689	3060	3319	3490	3158	0.0083	4.8484	2291	27.5451	1704	19.0620
19	30	A	293	530	686	932	1210	1695	2413	2824	3425	3351	3665	3372	0.0077	5.2286	2117	23.1975	1695	18.3285
20	30	A	298	504	657	864	1080	1529	2194	2600	3006	3231	3411	3113	0.0084	5.1802	2347	24.4572	2006	22.0592

Appendix Table 3. Radiographic bone aluminum equivalency (RBAE) max calculated values using aluminum step wedge with 1 to 11 steps. Values obtained from dorsal/palmar (DP) radiograph evaluating medial and lateral cortices of third metacarpal bone for each yearling horse at 60 d.

			Max. Value (INT)-The value of the highest intensity pixel in the volume Aluminum Step Wedge Steps 1 through 11 (mm)											Calculated Values						
Horse	Day	Vibration (A=Yes; B=No)	5	8	11	14	17	20	23	26	29	32	35	Difference first, last AL step	m	b	X-Medial Cortices	Y-Medial Cortices	X-Lateral Cortices	Y-Lateral Cortices
1	60	B	358	552	670	868	1077	1499	2152	2584	2945	3207	3413	3055	0.0086	4.9147	2335	23.0928	1494	17.7432
2	60	B	123	452	633	912	1177	1603	2232	2831	3153	3395	3622	3499	0.0078	5.8074	2082	24.5109	1772	19.5481
3	60	A	383	569	769	946	1214	1634	2261	2700	3009	3168	3387	3004	0.0088	4.0282	2360	25.1127	2574	26.5943
4	60	B	316	539	716	944	1110	1541	2226	2746	3093	3260	3699	3383	0.0081	5.1635	2687	21.9929	1822	19.8912
5	60	A	228	451	649	874	1061	1423	2061	2595	2938	3279	3640	3412	0.0081	5.8628	2286	26.7118	1859	20.9204
6	60	B	131	359	572	780	987	1280	1918	2509	2980	3305	3697	3566	0.0077	6.9583	2433	27.7745	1568	19.1056
7	60	B	378	590	700	843	1132	1622	2289	2563	3005	3216	3443	3065	0.0086	4.5843	2318	24.1810	2532	26.2899
8	60	B	307	533	675	814	1076	1532	2161	2588	2910	3214	3389	3082	0.0086	5.0714	2503	25.8816	2355	25.2144
9	60	A	325	523	704	877	1123	1573	2216	2623	2954	3224	3405	3080	0.0086	4.7811	2964	26.4661	2055	22.3809
10	60	A	292	484	650	848	1023	1318	1926	2562	2975	3284	3661	3369	0.0081	6.0726	1822	26.2305	1801	20.5769
11	60	A	263	477	633	832	1098	1520	2210	2649	3058	3135	3268	3005	0.0085	5.2677	2143	23.4093	1549	18.3808
12	60	A	297	508	711	892	1196	1622	2288	2832	3106	3356	3567	3270	0.0081	5.0773	2919	17.5567	2683	26.6927
13	60	B	288	496	705	895	1095	1587	2273	2712	2996	3275	3360	3072	0.0084	4.9832	2228	22.9687	1541	17.9163
14	60	B	406	569	766	984	1274	1808	2455	2759	3157	3320	3437	3031	0.0084	3.9420	2907	28.5709	1901	19.9816
15	60	B	375	521	647	854	1119	1547	2193	2608	2928	3201	3288	2913	0.0087	4.7410	2295	24.1367	2154	23.4925
16	60	B	333	549	742	869	1171	1628	2404	2778	3156	3305	3534	3201	0.0081	4.8816	2740	28.4998	1826	19.7171
17	60	A	287	513	656	891	1098	1635	2277	2677	3054	3413	3408	3121	0.0082	5.1928	2257	23.9686	1628	18.5118
18	60	A	350	538	715	902	1171	1631	2312	2804	3060	3319	3490	3140	0.0083	4.7720	2291	27.3904	1698	18.7888
19	60	A	293	553	744	932	1185	1695	2413	2824	3108	3355	3665	3372	0.0080	4.8624	2117	22.9594	1830	19.5357
20	60	A	307	527	679	895	1152	1529	2261	2737	3008	3251	3492	3185	0.0083	5.0145	2412	24.0512	2069	22.2065

Appendix Table 4. Radiographic bone aluminum equivalency (RBAE) max calculated values using aluminum step wedge with 1 to 11 steps. Values obtained from dorsal/palmar (DP) radiograph evaluating medial and lateral cortices of third metacarpal bone for each yearling horse at 90 d.

			Max. Value (INT)-The value of the highest intensity pixel in the volume Aluminum Step Wedge Steps 1 through 11 (mm)											Calculated Values						
Horse	Day	Vibration (A=Yes; B=No)	5	8	11	14	17	20	23	26	29	32	35	Difference first, last AL step	m	b	X-Medial Cortices	Y-Medial Cortices	X-Lateral Cortices	Y-Lateral Cortices
1	90	B	541	745	850	1106	1521	2057	2648	2951	3144	3357	3452	2911	0.0087	2.2672	2778	26.4885	2275	22.1029
2	90	B	553	733	826	1090	1437	1982	2608	3004	3167	3359	3416	2863	0.0086	2.6090	2515	24.3056	2367	23.0288
3	90	A	600	754	855	1128	1474	2013	2648	3021	3229	3327	3479	2879	0.0086	2.2908	3068	28.8200	2242	21.6775
4	90	B	531	684	880	1060	1458	2111	2748	3080	3265	3461	3530	2999	0.0082	2.9476	3065	28.1546	2396	22.6527
5	90	A	651	735	968	1243	1748	2377	2880	3187	3219	3361	3415	2764	0.0087	1.2658	2908	26.4621	2845	25.9163
6	90	B	512	666	794	1060	1455	2183	2648	2957	3157	3451	3445	2933	0.0084	2.9386	2570	24.5404	2153	21.0354
7	90	B	685	772	956	1191	1504	2172	2782	3050	3226	3284	3404	2719	0.0089	1.4466	2100	20.0596	2492	23.5341
8	90	B	613	742	981	1203	1638	2190	2763	3080	3279	3368	3534	2921	0.0087	1.5899	3065	28.1256	2368	22.0912
9	90	A	601	766	936	1114	1440	2012	2697	3098	3302	3445	3537	2936	0.0084	2.4696	3019	27.8386	2807	26.0571
10	90	A	527	684	830	996	1378	2066	2614	2924	3190	3325	3323	2796	0.0086	2.9289	2337	23.0070	2422	23.7373
11	90	A	537	688	813	1056	1445	2089	2600	2958	3178	3339	3355	2818	0.0086	2.6909	2928	27.9648	2512	24.3740
12	90	A	570	766	899	1101	1514	2007	2650	3058	3225	3379	3495	2925	0.0086	2.3079	2833	26.6345	2870	26.9523
13	90	B	621	815	936	1186	1568	2154	2847	3001	3243	3400	3462	2841	0.0087	1.6123	2933	27.1468	2798	25.9715
14	90	B	628	825	936	1217	1615	2184	2724	3045	3188	3280	3349	2721	0.0090	1.0886	3027	28.4772	1939	18.6329
15	90	B	588	797	1003	1211	1621	2229	2809	3170	3364	3523	3570	2982	0.0084	1.7959	3057	27.4249	2925	26.3183
16	90	B	644	871	1018	1278	1765	2381	2820	3095	3308	3413	3497	2853	0.0088	0.6525	3191	28.8433	2229	20.3445
17	90	A	578	771	911	1104	1531	2090	2758	3134	3252	3289	3472	2894	0.0085	2.2125	2703	25.3176	2142	20.5222
18	90	A	450	648	772	1059	1341	1890	2558	3004	3155	3217	3323	2873	0.0086	3.3246	2143	21.6787	1636	17.3364
19	90	A	555	673	879	1141	1462	2023	2695	3061	3175	3427	3575	3020	0.0084	2.6921	2973	27.6643	2757	25.8500
20	90	A	548	794	916	1209	1588	2220	2713	3045	3158	3225	3374	2826	0.0090	1.4518	2866	27.1100	2493	23.7707

Appendix Table 5. Radiographic bone aluminum equivalency (RBAE) max calculated values using aluminum step wedge with 1 to 11 steps. Values obtained from dorsal/palmar (DP) radiograph evaluating medial and lateral cortices of third metacarpal bone for each yearling horse at 120 d.

			Max. Value (INT)-The value of the highest intensity pixel in the volume Aluminum Step Wedge Steps 1 through 11 (mm)											Calculated Values						
Horse	Day	Vibration (A=Yes; B=No)	5	8	11	14	17	20	23	26	29	32	35	Difference first, last AL step	m	b	X-Medial Cortices	Y-Medial Cortices	X-Lateral Cortices	Y-Lateral Cortices
1	120	B	513	710	781	950	1199	1641	2368	2820	3114	3324	3397	2884	0.0086	3.7613	2001	20.9314	1606	17.5420
2	120	B	481	627	760	919	1153	1700	2271	2700	3133	3269	3489	3008	0.0085	4.1265	1914	20.4274	1655	18.2216
3	120	A	547	739	859	1045	1301	1885	2633	2947	3179	3407	3468	2921	0.0085	3.0550	2429	23.6254	2621	25.2513
4	120	B	634	761	888	1069	1464	2017	2651	3061	3238	3406	3561	2927	0.0085	2.4521	2976	27.7026	2009	19.4979
5	120	A	494	669	826	1021	1286	1864	2528	2869	3117	3371	3544	3050	0.0085	3.4032	2443	24.0621	1962	19.9946
6	120	B	580	755	888	1066	1339	1892	2493	3005	3214	3465	3566	2986	0.0084	2.9362	2206	21.5352	1713	17.3787
7	120	B	490	686	824	1054	1419	2054	2647	2891	3181	3366	3510	3020	0.0085	2.9547	2775	26.4746	1883	18.9143
8	120	B	545	723	843	978	1291	1831	2502	3008	3257	3359	3606	3061	0.0083	3.5265	2303	22.5450	2082	20.7200
9	120	A	560	736	788	957	1250	1778	2466	2939	3206	3360	3447	2887	0.0084	3.5523	2803	27.1541	1930	19.8033
10	120	A	579	740	919	1029	1340	1827	2510	2982	3229	3360	3434	2855	0.0086	2.8409	2090	20.8138	2215	21.8888
11	120	A	488	688	846	999	1263	1841	2354	2861	3250	3324	3406	2918	0.0086	3.4128	2424	24.1577	1758	18.4580
12	120	A	584	806	899	1110	1541	1971	2632	3067	3278	3359	3533	2949	0.0086	2.2147	3087	28.7263	3223	29.8943
13	120	B	582	769	886	1094	1530	2070	2693	3012	3215	3272	3538	2956	0.0087	2.1560	2661	25.2049	2416	23.0828
14	120	B	527	758	850	1070	1378	1859	2475	2888	3151	3340	3405	2878	0.0088	2.6581	2662	26.0582	2717	26.5417
15	120	B	509	702	848	997	1366	1962	2529	2906	3160	3384	3472	2963	0.0085	3.0625	2513	24.5053	2375	23.3278
16	120	B	451	617	745	942	1188	1623	2324	2792	3190	3353	3433	2982	0.0083	4.3359	2500	25.1880	2513	25.2964
17	120	A	500	685	834	1033	1264	1987	2441	2929	3176	3394	3444	2944	0.0085	3.2701	2103	21.1155	1561	16.5163
18	120	A	484	631	782	926	1153	1634	2312	2776	3007	3208	3404	2920	0.0087	3.9069	1976	21.1240	1500	16.9766
19	120	A	609	700	812	994	1286	1696	2390	2828	3118	3318	3432	2823	0.0087	3.1868	2354	23.7392	2214	22.5169
20	120	A	565	755	873	1057	1306	1787	2391	2976	3225	3399	3609	3044	0.0084	3.1892	2105	20.9285	1852	18.7964